Activity-based and targeted analyses of matrix metalloproteases

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Chapter 1

General introduction: matrix metalloproteases and their analyses in biological fluids

1. MMPs

1.1. Structure and classification

The study of proteolytic enzymes has been the field of extensive research in the past decades. Their involvement in many physiological and pathological processes and their defined structures make them ideal pharmaceutical targets in numerous areas such as oncology, inflammatory, and respiratory diseases [1].

Proteolytic enzymes are frequently subdivided into six groups according to their catalytic mechanism: aspartic, glutamic, metallo, cysteine, serine, and threonine proteases [2, 3]. However, glutamic proteases have never been reported within the mammalian family [1, 2]. Nucleophile attack is a common catalytic process of all proteolytic enzymes [3]. The aspartic, glutamic, and metalloprotease enzymes use a water molecule as nucleophile while the others (cysteine, serine, and threonine proteases) use an amino acid containing a nucleophilic side chain according to their respective name [1].

Metalloproteases enzymes, divided into the Zincin and the Dizincin groups are mostly zinc-dependent endopeptidases [3, 4]. Zincins use one catalytic Zn^{2+} ion while the Dizincins employ two catalytic ions [3]. The Zincin group includes the gluzincin, aspzincin, and metzincin clans. The last sub-group is characterized by the HExxH motif, which participates in complexing the catalytic Zn^{2+} ion as substantiated by numerous x-ray crystallographic studies of MMPs, notably by Bode *et al.* [5, 6]. Catalytic zinc ions are usually bound to three amino-acid residues and a water molecule while structural zinc ions complex with four amino acid residues but contain no water molecule in their coordination sphere [7]. Histidine residues of the conserved sequence HExxH motif serve as Zn^{2+} binding sites while the glutamic acid residue serves as a nucleophile which is required for the catalytic cleavage of the substrate. The third amino acid binding the catalytic zinc ion differs between the gluzincin, aspzincin, and metzincin proteases. The gluzincins and aspzincins employ glutamate and aspartate as their third Zn^{2+} binding ligand, respectively while metzincins were named after the presence of a methionine residue in the so-called "Met-Turn" forming a hydrophobic cleft around the catalytic Zn^{2+} ion [3, 4].

The Metzincin family, characterized by the consensus sequence HExxHxxGxxH, regroups the astacins, ADAMs/adamalysins/reprolysins, serralysins, matrix metalloproteases, snapalysins, leishmanolysins, and pappalysins [3, 4]. The distal histidine residue is brought in proximity to the catalytic Zn^{2+} ion via a hinge created by the glycine residue. Matrix Metalloproteases (MMPs), also called Matrixins [8], contain the consensus sequence HExxHxxGxxH and the methionine residue responsible for the 'Met-Turn' located eight amino-acid residues downstream. A typical MMP contains a signal peptide of~ 20 amino acids, a propeptide of ~ 80 amino acids and a catalytic domain of ~ 170 amino acids [3, 9, 10].

MMPs are secreted as pro-enzymes (also called zymogens).The pro-domain contains the consensus sequence PRCGxPD, where the cysteine residue binds to the catalytic Zn^{2+} ion via its

thiol moiety with the exception of MMP-23 [8]. This interaction is known as the cysteine switch with the thiol group occupying one place in the coordination sphere of the Zn^{2+} ion preventing water from binding [8, 10-12]. When the cysteine switch is opened, it is possible for water to interact with the catalytic Zn^{2+} ion which polarizes the water molecule to function as a nucleophile to catalyze hydrolysis of the scissile peptide bond of the substrate. In the most commonly described mechanism a glutamic acid residue acts as a general base by accepting a proton from the water molecule before transferring it to the substrate [3, 4, 9]. The catalytic domain of MMPs contains a second Zn^{2+} ion as well as 2 or 3 Ca^{2+} ions. These ions play a structural role and are important for the stability of MMPs [8].

The first MMP (MMP-1 a collagenase) in the mammalian kingdom was discovered in 1962 [13]. Today, 23 human MMPs are known and classified into six groups according to their structural organisation and/or their substrate specificity: collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP -10, MMP-11), matrilysins (MMP-7 and MMP-26), membrane type (MT)-MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24 and MMP-25) and others (MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27, MMP-28) (see **Table 1**) [3, 9-11, 14].

The signal peptide, the pro-domain and the catalytic domain are common elements of all MMPs (**Table 1**) [3, 9-11] while the hemopexin domain, at the C-terminal extremity, is shared by most MMPs. This domain, which is linked to the catalytic domain via a hinge region, is not present in the matrilysins (MMP-7 and MMP-26) and MMP-23. MMP-23 is unique since its C-terminus contains an immunoglobulin-like and a cysteine-array domain. The collagenases (MMP-1, MMP-8, and MMP-13) and gelatinases (MMP-2 and MMP-9) share a highly similar structure. However gelatinases contain three inserts in their catalytic domain (so-called fibronectin type II modules) which are essential elements for substrate recognition. Structural organization of MMP-11, MMP-21, and MMP-28 is also closely related to the collagenases and stromelysins. However, these enzymes possess a furin-like convertase cleavage site (RRKR or RxKR) in their pro-domain and constitute the "group of secreted convertase-activable MMPs" [3, 9, 10]. Such a domain is also found in the MT-MMP group. The MT-MMP group is subdivided into two further groups depending on how they insert into the cell membrane: the GPI-anchored MMPs (MT4-MMP and MT6-MMP) and the membrane associated MMPs (MT1-MMP, MT2-MMP, MT3-MMP, and MT5-MMP). The last group contains a type I transmembrane domain (MT1-MMP, MT2-MMP, MT3-MMP, and MT5-MMP) [3, 9-11].

Group	Members	Alternative name	Structural domains
Matrilysin	MMP-7 MMP-26	Matrilysin 2 Matrilysin 1	
Stromelysin	MMP-3 MMP-10 MMP-11	Stromelysin 1 Stromelysin 2 Stromelysin 3	
Collagenase	MMP-1 MMP-8 MMP-13	Interstitial collagenase; Collagenase 1 Neutrophil collagenase; Collagenase 2 Collagenase 3	
Gelatinase	MMP-2 MMP-9	Gelatinase A Gelatinase B	
Membrane type (MT)-MMPs	MMP-14 MMP-15 MMP-16 MMP-24	MT1-MMP MT2-MMP MT3-MMP MT5-MMP	
	MMP-17 MMP-25	MT4-MMP MT6-MMP	
Others	MMP-12 MMP-19 MMP-20 MMP-27	Macrophage elastase - Enamelysin -	
	MMP-23	CA-MMP	
	MMP-28 MMP-21	Epilysin -	

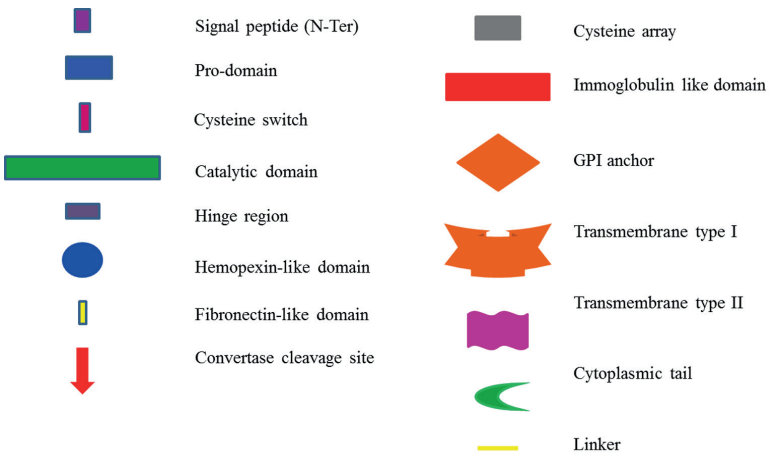

Table 1. Classification and structural organization of the 23 known human MMPs (adapted from Parks *et al.*[12] and Ugalde *et al.*[3]).

1.2. Regulation

The proteolytic activity of MMPs is regulated at multiple levels. Regulation of gene expression, compartmentalization, activation of the pro-enzymes, and finally inhibition by physiological inhibitors are all key steps through which these potent enzymes are controlled.

Expression of MMP genes is controlled by growth factors, hormones, cytokines, and physical stress. It is also known that cell-matrix and cell-cell interactions play a role in regulating the expression of MMP genes [8, 15, 16]. Most MMPs are secreted as pro-enzymes with latency maintained by the cysteine switch. A cysteine residue of the pro-domain, in the conserved sequence PRCGxPD, complexes the catalytic Zn^{2+} ion thus preventing access to the substrate and to a water molecule that participates in hydrolysis of the scissile peptide bond [10-12, 15, 17, 18]. Disruption of the cysteine switch is by consequence a fundamental phenomenon towards the activation of pro-MMPs [10-12, 15, 17, 18]. Activators of pro-MMPs may be other proteolytic enzymes (e.g the serine proteases trypsin, plasmin or other active MMPs) as well as chemical reactants (e.g. reactive oxygen species or SH-binding compounds such as APMA (4-aminophenylmercuric acetate) [18]. Two proteolytic activation pathways have been extensively studied and appear to be relevant *in vivo*. The first one is based on the activation of plasmin by tissue plasminogen activator (t-PA) or urokinase (u-PA) leading to the activation of MMPs, which can in-turn activate other MMPs. For example, activated MMP-1, MMP-10, MMP-3, MMP-2, and MMP-7 might activate pro-MMP-9 [18]. The second activation mechanism is initiated by furins, which cleave the pro-domain of MT-MMPs and MMP-1 inside the cytoplasm, which are capable of activating MMP-2 and MMP-13 which in turn can activate pro-MMP-9 [18].

Active MMPs are controlled by two types of endogenous inhibitors: α2-macroglobulin and tissue inhibitors of metalloproteases (TIMPs). The plasma glycoprotein, α_2-macroglobulin, is composed of four identical subunits with a total molecular weight of ~772 kDa [11, 19]. This protein is mainly synthetized in hepatocytes and inhibits about any endopeptidase through a generic mechanism [17]. Active MMPs cleave the so-called "bait region" of α_2-macroglobulin inducing a conformational change within the inhibitor leading to trapping of the active enzymes [17]. Inhibition of MMPs by α_2-macroglobulin does not block the catalytic site of the enzymes so that low-molecular weight substrates may still be cleaved while large protein substrates are excluded and not cleaved [17].

A second class of inhibitors, which is specific for MMPs, is represented by the TIMP family [8, 10, 11, 15, 16, 20]. The first TIMP was characterized in the 1970s [11, 21]. There are 4 TIMPs (TIMP-1 to TIMP-4) with a sequence homology of 42 – 52% [20] and with a molecular weight ranging from 21 to 30 kDa (184 to 194 amino acids). TIMPs inhibit all active forms of MMPs with inhibition constants in the low picomolar range, except for TIMP-1 which is a poor inhibitor of MMP-19, MT1-MMP, MT3-MMP, and MT5-MMP [10, 19]. TIMPs are slow-tight binding inhibitors that form a 1:1 complex with MMPs [20].

The N-terminal extremity of TIMPs is essential for inhibition, since this moiety by itself is sufficient for MMP inhibition [11]. Solving the crystal structure of the TIMP-1/MMP-3 complex in 1997 provided a more detailed insight into the inhibition mechanism [22]. The residues C_1TCV_4 and $E_{67}SVC_{70}$ (linked by a disulfide bond) enter into the active site of MMPs with C_1 binding the catalytic Zn^{2+} ion which inhibits the activity of the enzyme.

Other proteins possessing domains that are highly similar in structure and/or in sequence to TIMPs are known to inhibit MMPs. These comprise the C-terminal extremity of the procollagen proteinase enhancer protein (PCPE), the NC1 domain of collagen IV, the reversion-inducing protein with Kazal motifs (RCEK), and the tissue factor pathway inhibitor-2 (TFPI2) [3]. The internal region of TFPI2 has been shown to inhibit MMP-1, MMP-2, MMP-9, and MMP-13 [19].

1.3. Function

MMPs are involved in numerous physiological and pathological processes such as tissue repair, angiogenesis, host defense, tumor progression, or inflammation [12]. Vascular disease is also known to be linked to a disregulation of MMP activity as well as arthritis [11] or lung disease [14]. The involvement in physiological processes such as mammalian development, embryo implantation, morphogenesis, growth and wound healing are all well documented [11].

For many years the major function of MMPs has been attributed to the remodeling of extracellular matrix (ECM) proteins such as fibrillar and non-fibrillar collagens, gelatin, aggrecan, fibronectin, laminin, and elastin [3, 9, 10]. Collagenases are well known to be involved in the degradation of fibrillar collagens (such as collagen I, II, III, VII) [3, 9]. Remodeling of collagens by MMP-1, MMP-8, and MMP-13 leads to the formation of gelatin which might be further degraded by the gelatinases MMP-2 and MMP-9. Gelatinases are also known for their ability to degrade collagen IV and V. Stromelysins (MMP-3, MMP-10, MMP-11) show a broad substrate spectrum and the ability for cleaving numerous glycoproteoglycans. Membrane-associated MMPs (MT-MMPs) are known to be involved in the activation (cleavage of the pro-domain) of other MMPs [3, 9]. For example they can process pro-MMP-2 with the exception of MT4-MMP. MT-MMPs can also digest a number of ECM molecules, and MT1-MMP has collagenolytic activity on type I, II, and III collagens. The other MMPs show a very diverse substrate spectrum but are only present at specific locations. MMP-12, for example, is mainly secreted by macrophages with elastin as the main substrate. MMP-20 is found in newly formed tooth enamel [3, 9].

Over the last years the implication of MMPs in the processing of other biologically active molecules has become more and more evident [8, 10, 11, 23]. Chemokines, of which there are more than 50 members, are involved in the innate and acquired immune response [12]. It has been shown that a number of MMPs may be involved in controlling the activity of these small proteins directly (e.g. by cleavage) or indirectly by targeting other components which influence their activity [12]. Cytokines and growth factors such as TGF-β (Tumor Growth Factor-β) or proTNF-α (proTumor Necrosis Factor-α) are also substrates of MMPs [12, 23]. Growth factors are frequently associated with proteins of the extracellular matrix and remodeling of extracellular matrix proteins might thus release these messengers. Cleavage of these "non-traditional substrates" is involved in many processes such as cellular proliferation, apoptosis, cell migration, cell-cell communication, and tumor progression [23].

2. Analysis of MMPs in biological fluids: absolute and relative quantification

MMPs have been implicated in a wide range of diseases but their etiological role is often unclear. To shed more light on this point, it is necessary to quantify MMPs in a sensitive and specific manner [24, 25]. The quantification of MMPs is currently mainly performed by immunoassays (e.g. ELISA (Enzyme-linked immunosorbent assay)) although proteomics techniques might be a promising alternative [24, 25].

The first immunoassay, a Radio Immuno Assay (RIA), was described in 1960 by Berson and Yalow for the measurement of human insulin in plasma [26]. Due to problems linked to the use of radioactivity, Perlmann and Engvall developed the ELISA technique and the EIA (Immunosorbent Assay) technique was introduced by Schuurs and Weemen [26-28]. Since then, immunoassays have become the mainstay for absolute protein quantification in clinical research, diagnostics and the study of biomarkers.

Commercially available immunoassays are also widely used for the analysis of MMPs [29, 30]. Some manufacturers provide multiplex assay kits that allow measurement of a number of MMPs and sometimes also TIMPs simultaneously. ELISA assays remain up to date the most sensitive methods (pg/mL range) to measure proteins in biological samples. To develop a specific and sensitive ELISA in the most common 'sandwich' format, two antibodies recognizing different epitopes in the same protein with high specificity and affinity are required. The need for two 'orthogonal' high-affinity antibodies may render assay development costly and time consuming. It is furthermore uncertain whether the antibodies recognize the target protein specifically, since the readout of the assay is indirect (color, fluorescence, chemiluminescence).

Proteomics, based on the "shotgun approach", is mainly used for the discovery of proteins that may be involved in a given biological phenomenon. Most comparative proteomics studies rely on relative quantification strategies. In the shotgun approach, the samples of interest are proteolytically digested (most often with trypsin) before analysing the obtained peptides by liquid chromatography - mass-spectrometry [31]. The detected peptides might be used for relative protein quantification based on ion intensity (peak area/peak height) or spectral counting (label-free approaches) or relative quantification may be based on differential stable isotope labelling [32]. Fractionation and/or removal of the most abundant proteins are frequently required for detecting low abundant proteins [33]. An interesting example from this field is the study of Wu *et al*.[34]. Bronchoalveolar Lavage Fluid (BALF) (from asthma patients and healthy controls before and after a 24 h segmental allergen challenge) was ultrafiltered, depleted of the six most abundant proteins and analysed by SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) followed by in-gel trypsin digestion and analysed by LC-MS/MS analysis. About 1500 proteins were identified including low abundant protein such as chemokines, cytokines, complement factors and MMP-9. Furthermore there was a strong correlation between quantification by the proteomics approach and ELISA with a correlation coefficient of 0.9827 [34].

Targeted analysis by LC-MS/MS in the SRM (Selected Reaction Monitoring) mode is a well-known approach for small-molecule analysis but has only recently been introduced as a quantitative technique for proteins [25, 31]. Most SRM analyses are performed on triple quadrupole mass spectrometers. In this mode, the first and third quadruple are operated as ion filters for the selected peptides and fragments derived therefrom while the second quadrupole functions as a collision cell [25, 31]. Specificity of an SRM assay is due to a) the mass over the charge ratio differences between the parent and the product ions (so-called transitions), b) the retention time of the targeted peptides and their co-elution with an identical stable-isotope-labeled internal standard, and c) the ratio between the intensity of the different transitions.

In recent years, there has been an increasing interest in the use of liquid chromatography–tandem mass spectrometry (LC–MS/MS) in the SRM mode for protein quantification [24, 25, 31, 35-41]. One advantage of SRM-based protein assays is its facile multiplexing due to the capacity of modern triple quadrupole mass spectrometers to monitor a range of peptides in a single chromatographic run using scheduled SRM. However, the main drawback of this technique, compared to immunoassays, is its lower sensitivity. SRM-based protein quantification in plasma, without immunodepletion and/or extensive fractionation steps, allows protein detection at the low µg/mL level [42]. Immunoaffinity enrichment of proteins or target peptides allows to reach the low ng/mL to sub-ng/mL concentration range, however, at the expense of from similar drawbacks as immunoassays, which are mainly due to the need for time-consuming assay development.

Chapter 3 of this thesis shows an SRM assay for MMP-9 in BALF which does not require extensive fractionation or affinity-enrichment of the targeted protein while reaching the low ng/mL range [39].

3. Analysis of MMPs in biological fluids: activity-based assays

Quantification (absolute or relative) of the overall amount of MMPs in biological fluids does not take their activation status into consideration. However, pathological events are likely linked to the deregulation of MMP activity rather than simply a change in amount. Some attempts have been made to measure and quantify MMPs as well as their main inhibitors the TIMPs.

Activity of MMPs in biological fluids might be analyzed by two main approaches. The first is based on the use of substrates while the second is based on the use of inhibitors, termed 'substrate-based' and 'inhibitor-based' assays for the sake of this chapter. Substrate-based assays usually require radioactive or fluorescent measurements for monitoring the activity of MMPs while inhibitor-based assay make use of synthetic MMP inhibitors that target the active site and can be used to enrich or tag active proteases in an activity dependent manner. Assay that focus on studying MMP activity *in vivo* will be discussed in paragraph 4 of this chapter.

3.1. Substrate-based assays

One of the first attempts to measure MMP activity was reported by Tint *et al.* in 1961 [43] by monitoring the change in weight of collagen that was incubated with collagenases showing a correlation between collagenase concentration and the degradation of the collagen substrate. Since then, numerous substrate-based procedures have been developed to follow MMP activity. The assays described below are a summary of the most common techniques. The reviews of Kriskova *et al.*, Lombard *et al.*, and Cheng *et al.* give a broader overview [29, 30, 44].

In the earliest attempt, radiolabeling of natural substrates has been extensively used. Radiolabeled substrates such as casein, collagen, and gelatin were incubated with the samples of interest and cleavage products were separated from non-digested proteins by protein precipitation. Measurement of the released radioactivity in the supernatant was thus directly proportional to enzyme activity [44, 45]. Another strategy was proposed by Manicourt *et al* [45]. Radiolabeled substrates (proteoglycans, gelatin, casein) are derivatized with [^{3}H] acetic anhydride and immobilized on a microtiter plate. This design allows screening a large number of samples [45]. Later on extensive development of analytical techniques has been pursued for solving the problems linked to the use of radioactivity. For example a substrate-based assay was proposed by Gray *et al.*[46]. Activity of purified collagenase was followed by monitoring the degradation products of DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-DArg by HPLC measurement.

Gelatin-based assays are a diverse set of assays applied to MMPs. One of the first assay formats used immobilized biotinylated gelatin (labeled with biotinyl-*N*-hydroxysuccinimide ester) was proposed by Koristas *et al.*[47]. The biotinylated protein was immobilized in microtiter plates and proteolytic activity of, for example papain, pepsin, thermolysin, and trypsin, were measured by incubation with streptavidin-alkaline phosphatase to quantify the biotin-gelatin complex remaining after proteolysis. Paemen *et al.* used a similar approach with biotinylated gelatin bound to avidin-coated microtiter plates as substrate for assessing the activity of tetracycline inhibitors on gelatinase B (MMP-9) [48]. A slightly different approach (with immobilized streptavidin) was developed by Ratnikov *et al.* for studying of MMP-9 and MMP-2 activity [49].

Gelatin zymography is currently the most widely used and the most sensitive assay for MMP-2 and MMP-9 analysis[50]. To this end, gelatin is co-polymerized in the polyacrylamide gel prior to performing protein separation by SDS-PAGE. Refolding of MMPs in the polyacrylamide gel after electrophoresis is performed by Triton washes before incubating the gel in assay buffer to develop the zymogram. With this strategy all forms of MMP-2 and MMP-9 are detected: active MMPs, pro-MMPs (which might be distinguished from the active ones based on molecular weight) and TIMP-MMP complexes due to the denaturation-renaturation step. One of the first gelatin zymograms was reported in 1980 by Heussen *et al.* [51]. Gelatin zymography allows detecting MMP-2 and MMP-9 at the low pg range [52]. MMP-1, MMP-8 and MMP-13 can also be detected on by gelatin zymography but with lower sensitivity. Alternative strategies, less used and less sensitive, are based on generic protein substrates such as collagen for monitoring collagenases (MMP-1, MMP-8, MMP-13) [53 54].

An innovative and very sensitive assay is based on the use of an engineered substrate, modified pro-urokinase, as developed by Verheijen *et al.*[55]. Pro-urokinase was modified with the peptide sequence RPLGIIGG which is a target of active MMPs. Cleavage of the engineered pro-urokinase by MMPs within biological samples is directly proportional to the activation of urokinase [55]. Hanemaaijer *et al.* adapted this principle to develop a specific assay for MMP-9 activity, with a sensitivity similar to gelatin zymography, by combining it with an immuno-capture step [56].

An easy but not very specific assay format relies on proteolytic cleavage of profluorescent peptide substrates. The substrate peptides contain a fluorescent group which is internally quenched (e.g. by a proximate dinitrophenyl group). Cleavage of the substrate by active MMPs releases quenching since quencher and fluorophore are located in the N- and C-terminal part of the cleavage products, respectively, leading to an increase of the fluorescent measurement. This assay is a very useful tool for monitoring the activity of purified proteases.

3.2. Inhibitor-based assays

An efficient synthetic MMP inhibitor should contain a moiety that is able to complex the catalytic Zn^{2+} ion (ZBG) and a structural motive that is able to interact with the catalytic site of the active enzyme, notably with the different sub-pockets of MMPs. The main common ZBGs are carboxylates, aminocarboxylates, sulfhydryls, derivatives of phosphoric acid, thiolates and hydroxamates [41, 57, 58].

On the left-handed and right-handed side of the catalytic Zn^{2+} ion three pockets are commonly described (S3-S2-S1 and S1′-S2′-S3′) [57]. MMP inhibitors are commonly described following this nomenclature. The inhibitors which interact with the non-primed site (the left-handed or P1-P3 site) have rather low affinity. However, the commercially available inhibitor Pro-Leu-Ala-NHOH with such a mode of inhibition is frequently used despite its modest IC_{50} in the μM range [41]. Inhibitors interacting with the primed site (P1′-P3′) generally show IC_{50} values in the nM range. Finally a third class of inhibitors is represented by a structure spanning both the non-primed and the primed site [41].

Despite initially promising results, MMP inhibitors have widely failed as pharmaceutical drugs in the fields of oncology and inflammatory diseases (see Dove *et al.*, for a review) [58] due to severe side effects [57-59]. The main reason of these side effects is probably due to the lack of enzyme specificity and the pleiotropic roles of MMPs, for example in tissue regeneration. There have thus been considerable efforts to increase enzyme specificity by designing inhibitors based on 3-dimensional structures of enzyme-inhibitor complexes. The S1′ pocket varies the most between members of the MMP family in terms of structure and remains the most targeted site to gain specificity for synthetic inhibitors [57, 59-61]. The design of more specific inhibitors has been reviewed extensively in a number of publications [57, 59-61].

While success with synthetic MMP inhibitors in clinical applications has been limited, there are a number of analytical techniques that are based on derivatives of broad-spectrum synthetic MMP inhibitors. One area relates to so-called activity-based probes (ABP) and another to the use of inhibitors as affinity ligands in activity-based enrichment strategies. Both techniques target active

MMPs in a family-wide manner, for example in biological fluids, cells in culture or tissue, before proceeding to downstream analyses that deconvolute the spectrum of targeted proteases (e.g. SDS PAGE followed by mass spectrometric identification). These techniques may help in gaining a better understanding of the roles of MMPs in biological mechanisms and disease states since they are activity-dependent and thus provide insights into which MMPs should be targeted under which conditions for developing inhibitors with better pharmacological properties.

The ABP strategy is based on the use of synthetic probes carrying three functional moieties (an element to recognize the active site of the active enzyme, a reactive group to label the targeted protein covalently, a reporter-tag to enrich and detect the labeled enzyme) [62-64]. This design has been successfully applied to other enzyme families such as the cysteine proteases, the serine hydrolases, and proteasome subunits [65, 66]. In the case of serine hydrolases or cysteine proteases labeling is based on the use of an electrophilic group in the probe that reacts with a nucleophilic functional group in the enzyme's active site (e.g. the hydroxyl group of serine or the thiol group of cysteine) to form a covalent bond. The catalytic properties of MMPs, which are based on the activation of a water molecule in the active site through interaction with a Zn^{2+} ion, prevent such an approach. A photoreactive group (aryl azide, diazirine or benzophenone) carried by the probe is needed to introduce a covalent bond with the targeted protein upon irradiation. This ABP technique has been investigated in several studies for a panel of MMPs demonstrating labeling of active enzymes but not of the pro-forms or TIMP-enzyme complexes This approach may also be used to study a panel of MMPs. However, labeling is never complete and the efficiency of labeling varies considerably between different MMPs. Dive *et al.* reported a labeling efficiency of ~1% for MMP-8 while MMP-12 was labeled to ~40% [67, 68]. Such efficiency differences make comparative, quantitative studies concerning the activation and inhibition of MMPs in complex biological samples difficult even if compensated for by the inclusion of stable-isotope-labeled internal standards. To tackle this problem, Cravatt *et al.* synthetized a library of ~20 synthetic probes (a so-called cocktail of probes) where the tag was added to the covalently labeled MMPs in a second step using click chemistry to avoid a negative effect of the tag on the inhibition and labeling of active enzymes [69]. While spiked MMPs were detected down to 100 ng/mg total protein in a lysate of mouse liver (10 ng/mg for MMP-1), no endogenous, active MMPs were detected in the studied human cancer cell lines (MUM-2B/MUM-2C). However, detection of other active members of the metalloprotease family such as ADAM-10, ADAM-17, AlaAp, Neprilysin and AFG3 were shown [69]. It should be noted that all of these enzymes are membrane proteins and that no soluble, active matrix metalloproteases were tagged.

The work of Bregant *et al.* compared the ABP technique to the enrichment of spiked, active MMPs that had interacted with a biotinylated inhibitor via streptavidin affinity chromatography [70]. Two different probes, one with and one without a photoactivatable group, were tested to compare enrichment of active MMPs. Spiked samples were first incubated with the biotinylated, synthetic inhibitors until full inhibition of the added active MMPs was reached, as judged by fluorescent measurements, before proceeding to the enrichment of the inhibitor-MMP complexes. The amount

of the probes was adjusted according to their respective IC_{50} values. While affinity enrichment of the spiked enzymes was almost complete, photoaffinity capture was only partial, since not all of the added active enzymes were labeled. This comparative study highlights the benefit of the affinity enrichment approach over the ABP strategy.

Further advantages of the activity-based enrichment versus the ABP strategy were highlighted by Freije *et al.*[71] one being that immobilized inhibitors can be recycled thus avoiding the use of considerable amounts of synthetic probes as for the ABP strategy. The work of Freije *et al.* demonstrated further the benefit of using immobilized inhibitors for the automated enrichment of active MMPs in view of future clinical studies [72, 73]. The activity-based enrichment strategy allows moreover the analysis of multiple active MMPs in parallel (multiplexing) as shown for MMP-1, MMP-7, MMP-8, MMP-10, and MMP-13, which were all enriched to more than 90% [72].

Promising results of the activity-based enrichment strategy for the detection of active endogenous enzymes have been previously shown. Freije *et al.* showed enrichment of MMP-9 from synovial fluid by gelatin zymography [72]. The work of Hesek *et al.* using a similar strategy, demonstrated its multiplexing capacity by analyzing MMP-2, MMP-1, and MMP-14 [74]. Enrichment of MMP-2 and MMP-14 from carcinoma tissue extracts was shown by zymography and western blot analysis, respectively. The work of Geurink *et al.* revealed the enrichment of ADAM-17 from PMA (phorbol-12-myristate-13-acetate)-stimulated cultured A549 cells by western blotting [75].

4. Analysis of *in vivo* MMP substrates

Substrate-based assays have been, and still are, extensively used for monitoring MMP activity in biological fluids but they do not provide insight into potential *in vivo* substrates of these enzymes. In general, it is very difficult to assign true and biologically relevant *in vivo* substrates to a given enzyme, due to the often artificial circumstances of measuring proteolytic activity. However, there have been recent advances, which bring us a step closer to discriminating artefacts from true biological activity. One way to do so is to look directly for the degradation products under different conditions.

Interesting work has been published by Nemirovskiy *et al.* and by Li *et al.* in this field [76, 77]. Degradation of type II collagen has been associated with osteoarthritis (OA) [76, 77]. Degradation products of type II collagen might thus be an indicator of the progress and state of OA. [77].. Based on this idea, an immunoaffinity LC–MS/MS assay has been developed. A 45-mer peptide (uTIINE) was shown to be the most abundant degradation product of type II collagen by MMP-13. Li *et al.* validated the developed assay and applied it to urine samples [76]. The assay was used to study the biomarker in a comparison between age- and sex-matched normal subjects, patients with confirmed radiographic OA and subjects with symptoms of OA that were confirmed by radiographic measurements. The findings suggest that a change in uTIINE concentrations within a subject over time is a better biomarker for disease progression than comparison of absolute levels between normal and OA subjects.

The work of Nemirovskiy *et al.* and Li *et al.* was based on a known substrate of MMP-13. A different approach to study the degradation products of MMPs is based on the COFRADIC (Combined Fractional Diagonal Chromatography) method. The COFRADIC technique was pioneered by the group of Gevaert *et al.* for reducing proteome complexity [78, 79]. This strategy is based on chemical modifications of specific amino acids, which lead to retention time shifts when reanalysing the same sample on the identical chromatographic system. One of the first reactive amino acids tested for this assay was the methionine residue. The oxidation of methionine to its sulfoxide by hydrogen peroxide leads to a more hydrophilic peptide which is easily separated from the non-modified peptides by reverse-phase HPLC. The COFRADIC technique was later extended to other amino acids as well as to analysing the N-terminus of proteins [79]. To this end proteins are first reduced and alkylated prior to acetylation of all available primary amines (α- and ε-amines) with sulfo-*N*-hydroxysuccinimide acetate. Tryptic digestion results in cleavage only after arginine residues thus creating a range of new N-termini. Treatment with 2,4,6-trinitrobenzenesulfonic acid will modify internal peptides (peptides with new N-termini) by introducing a hydrophobic trinitrophenyl group. These hydrophobic peptides are easily separated from the more hydrophilic, acetylated, N-terminal peptides [79]. Analysis of the ensemble of N-terminal peptides under different conditions can provide insights about protein targets of ongoing proteolytic processes.

The group of Overall used an innovative strategy for characterizing the N-termini of proteins and applied the developed technique to the study of MMPs [80, 81]. The approach is based on a dendritic polyglycerol aldehyde polymer (HPG-ALD) and compares a control sample with a sample where proteolysis occurred. All primary amines in each sample are labelled with (d(2)C13)-formaldehyde (heavy) or (d(0)C12)-formaldehyde (light) catalysed by sodium cyanoborohydride resulting in dimethylation of the amines. The labeled proteomes are combined and trypsin-digested before being incubated with the HPG-ALD polymer, which captures all free amines that were generated during trypsin digestion. Unbound peptides comprising dimethylated lysines, acetylated, cyclized and isotopically labeled protein N-terminal peptides and the neo-N-terminal peptides product are recovered by centrifugation, and analysed by high-mass-accuracy tandem mass spectrometry.

Characterization of neo- N-termini, due to a specific proteolytic event, is based on measuring the abundance ratio of the peptides between the control sample and a sample where protease-specific cleavage occurred. If a protein is a substrate for an enzyme, peptides might be either generated (increased protease/control ratio) or lost (decreased protease/control ratio).[80, 81]. This approach, named terminal amine isotopic labeling of substrates (TAILS), was slightly modified and adapted by stable-isotope labelling with a 4-plex iTRAQ kit which allows analysing 4 samples in parallel [81].

5. Scope of the thesis

Since the discovery of the first animal collagenase more than 50 years ago, analytical tools to monitor the activity of MMPs have been extensively researched. Knowledge in chemistry, biology, biochemistry and molecular modeling was needed to assess the involvement of these enzymes

in many different pathological processes. Recent analytical techniques for studying MMP activity might be classified in two main groups, one trying to measure enzyme activity directly while the other investigating the products of the reaction.

Synthetic inhibitors of MMPs have been widely investigated as potential pharmaceutical drugs with a focus on oncology and inflammatory diseases but with rather disappointing results. However, application of synthetic inhibitors for analytical purposes looks promising [58].

The overall goal of this thesis was the design of innovative analytical techniques, with a particular focus on targeted analysis by LC-MS/MS, for monitoring MMP activity in biological fluids.

Chapter 2 of this thesis describes a photoactivatable, biotinylated, activity-based probe that has previously been shown to label MMPs more efficiently in comparison to earlier versions of this probe [75]. In this chapter we show that labeling occurs with a 1:1 stochiometry by mass-spectrometry analysis. Enrichment of the labeled protein by avidin-affinity chromatography proved to be efficient and selective.

Chapter 3 describes an SRM assay applied to MMP-9 in Bronchoalveolar Lavage Fluid (BALF). Many targeted analyses have foccused on serum while BALF is more suitable for studying pulmonary diseases. The MMP-9 assay is based on two critical steps: methanol-chloroform precipitation and LC-MS/MS analysis. Methanol-chloroform precipitation allows desalting and cleaning of the samples (e.g. removing the abundant phospholipids), concentrating and denaturing proteins before proceeding to their reduction/alkylation and trypsin digestion in the presence of labeled internal standard peptides. The second critical step is development of the targeted analysis by HPLC-MS/MS including optimization of the gradient, the resolution of the first and last quadrupole mass analyzer as well as reducing carry-over between runs. The developed SRM assay allows the absolute quantification of MMP-9 down to the low ng/mL level. The developed assay was adapted to MMP-12 by monitoring a different set of signature peptides. However, MMP-12 could not be detected in BALF.

Since activity of MMP-9 is not monitored using this assay, we combined the SRM assay with the activity-based enrichment approach focusing on MMP-9 and MMP-12 and this is reported in **Chapter 4**. We further extended this methodology by monitoring peptides belonging to the pro-domain of MMP-9 as well as the main endogenous inhibitor of MMP-9, TIMP-1.

Finally **Chapter 5** discusses the limits and advantages of the developed techniques in view of their extension to other enzymes and their potential for automation in view of analyzing larger series of clinical samples.

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Chapter 2

Activity-based probes for labeling MMPs

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Abstract

Profiling active matrix metalloproteases (MMPs) in biological samples is challenging, since the enzymes do not have a nucleophilic, reactive amino acid side chain in their active site. Photoactivatable probes have proven successful in overcoming this limitation but widespread use is hampered due to low and variable labeling yields. In this study we show that probes containing a succinyl hydroxamate motif and a trifluoromethylphenyldiazirine photoreactive group label the catalytic domain of human recombinant MMP12 with a 1:1 stoichiometry and allow to enrich active MMP12 on monomeric avidin beads after labeling with a biotin-containing probe. Characterization of the amino-acid(s) involved in labeling is still under investigation.

1. Introduction

Matrix metalloproteases (MMPs), belonging to the family of zinc-dependent endopeptidases, are involved in a wide range of biological processes related to extracellular matrix (ECM) remodeling and tissue resorption [1-5]. The proteolytic activity of MMPs is tightly regulated *in vivo* at multiple levels including gene expression, extracellular and/or cellular compartmentalization of the enzyme, activation of the proenzyme (zymogen form), and inhibition of the active enzyme by α_2-macroglobulin and tissue inhibitors of metalloproteases (TIMPs). Any deregulation of MMP activity may lead to a wide range of pathological processes, as observed in diseases that have a component of chronic inflammation (e.g. rheumatoid arthritis or chronic obstructive pulmonary disease) [1-5].

Due to the tight regulation of MMP activity, measurement of active MMPs is more meaningful for the characterization of biological or pathological processes than determination of the overall abundance. There is thus a critical need for simple and robust methods for MMP analysis, which allow screening the activity in a broad family-wide manner followed by identification of the individual members (e.g. by mass spectrometry or immunochemical analysis).

There have been numerous efforts to create simple and robust assays for characterizing the activity of MMPs based on the use of synthetic inhibitors. Activity-based enrichment (see **Chapter 4**) gave promising results for the detection of active or activated endogenous enzymes. Another strategy based on the use of synthetic inhibitors is the activity based probe procedure (ABP) which has been studied extensively for other types of enzymes.

The most common and sensitive current methods for MMP activity analysis (zymography [1, 5, 6] and activity-dependent ELISA assays [7, 8]) are not suitable for a broad screen of MMP activity. While zymography allows a distinction between pro and active enzyme based on the molecular weight difference, it cannot distinguish the active and the TIMP-inhibited forms. Furthermore, it is known that pro-MMPs may be activated during refolding in the zymography gel and that TIMP-MMP complexes can dissociate during SDS-PAGE prior to zymography thus resulting in artificially elevated levels of apparently active MMPs in the sample. Zymography is not a very robust quantitative methodology, since detection sensitivity varies significantly between studies. Finally, zymography does not allow screening of all MMP family members, as detection depends on the substrate specificity of the respective enzyme and it does not allow identification of the respective enzyme. Activity-dependent ELISA assays are costly and specificity relies on antibody-enzyme recognition. It is uncertain whether the antibodies recognize the target protein specifically and it is difficult to rule out if other proteins contribute to the readout of the assay (color, fluorescence, chemiluminescence). It is furthermore not a family-wide screening methodology.

A more recently developed approach that is based on activity based probes (ABPs) has shown promise in the case of serine hydrolases, cysteine proteases and the proteasome [9-12]. Only a few reports have, however, been directed at MMPs, since these enzymes do not contain a nucleophilic amino acid side chain in the active site but rather activate a bound water molecule through interaction with a complexed Zn^{2+} ion [2, 4]. To label MMPs in an activity-dependent manner, three

critical and essential parts are required: a moiety targeting and recognizing the catalytic site of the active enzyme, a photocrosslinking group to form a covalent bond with the active enzyme, and a reporter tag for further analysis. The ABP technique has the additional benefit to allow purification of the labeled enzyme from a complex biological matrix based on an incorporated affinity tag, such as biotin, for further identification of the protein target [13, 14].

Application of the ABP technique to a panel of MMPs has been investigated by several groups demonstrating labeling of active enzymes but not of the zymogen forms nor of the TIMP-MMP complexes. However, labeling efficiencies varied widely from ~1% for MMP-8 to ~40% for MMP-12 as reported by Dive *et al.*[15].

Improvement of the ABP strategy requires more efficient synthetic probes. Thus a better understanding of the interaction between the probe and the enzyme is a crucial step. Determining the amino-acid(s) implied in the covalent bond might aid in designing more efficient ABPs and to reduce differences in labeling efficiency from one enzyme to the other. A previous report from our laboratory described two probes based on the succinyl hydroxamate motif differing in the position of the trifluoromethylphenyldiazirine photoreactive group [16]. It was demonstrated that directing the photoactivatable group towards the S1' pocket of MMPs resulted in more effective, family-wide labeling than directing the corresponding photoactivatable group towards the S2' pocket. In this Chapter we studied protein labeling of the S1'-directed probe in greater detail and demonstrate that labeling results in a 1:1 ratio between probe and enzyme when targeting MMP-12 CD (recombinant catalytic domain). We show furthermore that the labeled protein can be enriched with monomeric avidin beads, which resulted in better yields than enrichment on streptavidin beads.

Identifying the amino-acid(s) involved in the covalent bond with the synthetic probes requires enzymatic digestion (e.g. tryptic digestion) of the labeled enzyme before isolating the modified peptides. Currently we are able to detect the labeled peptide by Western Blot after separation of the digest on a Tris-Tricine-SDS-PAGE but identification of the labeling sites remains to be done.

2. Material and methods

2.1. Material

The expression vector pet21a including the sequence G_{106} to N_{268} catalytic domain plus the initiator M (via NdeI / NotI) of MMP-12 CD was obtained from GENEART (Regensburg, Germany). Ammonium bicarbonate (AB), dimethyl sulfoxide (DMSO), chloroform, dithiothreitol (DTT), formic acid (FA), calcium chloride, ammonium persulfate, sodium acetate, and iodoacetamide (IAA) were purchased from Sigma Aldrich. Trypsin (sequencing grade, cat. n° V5111) was from Promega, NaCl, glacial acetic acid, sodium thiosulfate, and sodium carbonate were obtained from Merck. Methanol and acetonitrile (ACN) were purchased from Biosolve and formaldehyde was from JT Baker. Sodium dodecyl sulfate (SDS), tris, glycine, ethylenediaminetetraacetic acid (EDTA), and silver nitrate were purchased from Duchefa, Brij-35 was from Janssen Chemica, and glycerol from Genfarma BV. 30%

acrylamide/bis-acrylamide (29:1) solution, bromophenol blue, and tetramethylethylenediamine (TEMED) were from BioRad. Monomeric Avidin agarose, trifluoroacetic acid (TFA) and Zeba Spin desalting columns (cut-off: 7000 Da) were purchased from Pierce (Pierce, cat. n° 89882). Ultrapure water (resistivity of 18.2 MΩ·cm) was obtained from a Sartorius Stedim water purification system (model 611 VF). Ultra low molecular weight marker was purchased from Sigma (M3546), Precision Plus protein standards dual color from BioRad (161-0374), and the BenchMark protein ladder from Invitrogen (10747-012).

2.2. Synthesis of the probes

Probe 1 and Probe 2 (see **Figures 1A** and **1B** for chemical structures) including a succinyl hydroxamate motif and a trifluoromethylphenyldiazirine photoreactive group were synthetized as previously reported [16].

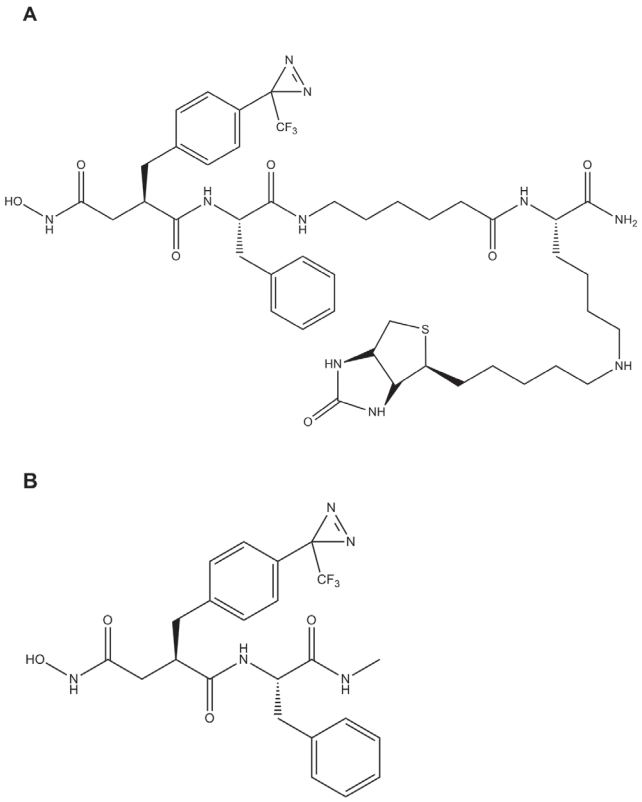

Figure 1. Structure of the photoactivatable inhibitors containing a photoactivatable trifluoromethyldiazirine group in the P1′ position targeting the S1′ pocket of MMP-12 CD. A) probe with a biotin moiety serving as tag to enrich the labeled protein via monomeric avidin beads (Probe 1); B) probe without biotin moiety used for the study of the stoichiometry ratio of the photolabeling (Probe 2).

2.3. Expression of recombinant MMP-12 CD

The catalytic domain of recombinant human MMP-12 was expressed and purified according the protocol of Parkar *et al.*[17]. The expression vector pet21a including the sequence G_{106} to N_{268} (via NdeI / NotI) of MMP-12 CD (with a Methionine residue at the N-terminal extremity) was obtained from GENEART (Regensburg, Germany). The 163-residue catalytic domain of MMP-12 CD was expressed in *E. coli* BL21 cells. The vector sequence was verified (Macrogen; http://dna.macrogen.com/eng/) from two different colonies after transformation.

Cells were cultured in baffled shake flasks at 37°C in Luria broth medium supplemented with ampicillin. Protein expression was induced at an optical density (OD600) of 0.5 by the addition of isopropyl beta-D-thiogalactoside (IPTG) to a final concentration of 1 mM. Cells were harvested by centrifugation and resuspended in 160 mL (for 2 liters of culture) of 100 mM Tris-HCl, pH8.0, supplemented by 4 tablets of protease inhibitors (EDTA-free; Roche). Deoxycholate was added to 0.1 % and the resuspended cells were incubated for 10 min on ice. After adding magnesium chloride and DNAse I to 40 mM and 5 µg/mL, respectively, the cells were lysed by sonication (3-times for 1 min; with a 1 min pause in between). The homogenized cell lysate was centrifuged and the insoluble material was washed as follows : 160 mL 0.1 % (v/v) Triton X-100, followed by 160 mL of demineralized water and finally with 10 mL of butan-1-ol. The inclusion bodies were resuspended in 10 mL (for 2 L of culture) of 8 M guanidinium-HCl, 50 mM Tris-HCl pH 8, 30 mM DTT (1h at 37°C).

The resolubilized, filtered inclusion bodies (1.2mL / 2.1 mg) were loaded on a size exclusion chromatography column (HiLoad Superdex 200 16/60 from GE Healthcare; 120mL column volume) that was equilibrated with 6 M guanidinium–HCl, 20 mM Tris-HCl, pH 8.0 at a flow rate of 1 mL/min (detection at 280nm) to remove large aggregates and interfering substances (ÄKTAprime plus system).

Fractions containing MMP-12 CD (verified by SDS-PAGE and MALDI-TOF-MS analysis) were subjected to refolding in two steps. Initially fractions were dialyzed (48 h at 4°C against 8 volume equivalents) of A) 3 M urea, 50 mM Hepes, 10 mM $CaCl_2$, 30 mM NaCl, 0.1 mM Zinc (II) acetate, pH 7.2 and then of B) 3 M urea, 10 mM Hepes, 2 mM $CaCl_2$, pH 7.0 (three buffer changes, 24 h each). MMP-12 CD autodegradation fragments were removed from the samples during the second refolding step on a strong cation exchange resin (SP-Sepharose fast flow, GE Healthcare).

The resin (2 mL) was first washed with 3 M urea, 10 mM Hepes, 2 mM $CaCl_2$, pH 7.0 before being incubated for 1 h at room temperature with 20 mL of the dialyzed protein at 0.1 mg/mL in 3 M urea, 10 mM Hepes, 2 mM $CaCl_2$, pH 7.0. After incubation with the samples, the resin was packed manually into a P10 column. Final refolding of MMP-12 CD was completed by gradually reducing the urea concentration in the following steps: 3 washes (one wash corresponds to 10 column volumes) with 2 M urea, 10 mM Hepes, 2 mM $CaCl_2$, pH 7.0, one wash with 1 M urea, 10 mM Hepes, 2 mM $CaCl_2$, pH 7.0, two washes with 10 mM Hepes, 2 mM $CaCl_2$, pH 7.0. Active MMP-12 was eluted with 10 mM Hepes, 2 mM $CaCl_2$, 250 mM NaCl, pH 7.0.

2.4. Labeling of MMP-12 CD

1.5 µg (~75 pmol) MMP-12 CD were incubated for 10 min in the dark at 4º C with a 2-fold molar excess of the Probe 1 or Probe 2 (unless otherwise mentioned**)** in 30 µL of 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM $CaCl_2$, 0.005 % Brij 35 and labeled by irradiation at 366 nm for 30 min (Camag universal UV lamp (20 W), distance to the plate of 4 cm in 96-well plates (Costar White)) at room temperature. Salt and detergent were removed prior to mass spectrometric analysis by methanol-chloroform precipitation according to the protocol of Wessel and Flugge [18]. After photoaffinity labeling the protein solution was mixed with 600 µL methanol, 150 µL chloroform and 450 µL water by vortexing and centrifuged at 13600 g for 5 min. The pellet was washed with 450 µL of methanol and centrifuged as above. The final pellet was dried in a vacuum concentrator centrifuge prior to further analysis.

2.5. Analysis by liquid chromatography – mass spectrometry (LC-MS)

Photolabeled MMP-12 CD was analyzed by nanoLC-MS using a microfluidics (chip-cube) interface (Agilent, cat. n° G4240A) on a C8 custom-made chip (Agilent, cat. n º G4240-63001 batch SPQ105) with a 40 nL enrichment column (Zorbax 300SB C-8, 5 µm) and a 75 µm × 43 mm separation column packed with the same chromatographic material. The interface contained a nanoelectrospray tip (2 mm length with conical shape: 100 µm OD × 6 µm ID) that was coupled on-line to an ion-trap mass spectrometer (MSD Trap-SL, Agilent). Photolabeled MMP-12 CD (1.5 µg) was dissolved in 0.1 % aqueous formic acid at a concentration of 0.1 µg/µL and 3 µL were loaded on the trap column in 0.1 % aqueous formic acid at 3 µL/min via the autosampler (Agilent, cat. no. G1389A) equipped with an injection loop of 8 µL and a thermostated cooler maintaining the samples in the autosampler at 4°C. The interface was connected to an Agilent 1100 series HPLC system containing the following additional modules: nanopump (cat. no. G2226A), capillary pump (cat. no. G1376A) and solvent degasser. The samples were enriched for 5 min followed by washing in the forward flush mode for another 5 min (0.1% aq. formic acid, 3% acetonitrile, 3 µL/min) after which the trapping column was switched in-line with the analytical column. MMP-12 CD was eluted with eluents A (0.1% aq. formic acid) and B (0.1% formic acid in acetonitrile) at a flow-rate of 0.25 µL/min using the following program: 5 min with 3% eluent A, a linear gradient from 3 to 60% eluent B at 0.9%/min followed by a gradient from 60 to 90% eluent B at 10%/min. Eluent B (90%) was maintained for 2 min to clean the column. Before each injection, the in-line trap and the analytical column were equilibrated with eluent A for 10 min at 3 and 0.25 µL/ min, respectively.

2.6. Analysis by MALDI-TOF (MS)

The protein pellet (~2 µg) of the methanol-chloroform precipitation was resuspended with 10 µL of Sinapinic Acid (SA) (10 mg/mL in 50% ACN 0.1% TFA). 2 µL were spotted on the target plate for

MALDI-TOF-MS analysis (Voyager DE-PRO, Applied Biosystems). The instrument was calibrated with a protein calibrant mix from Bruker Daltonics (No. 206355).
The MALDI-TOF-MS mass spectrometer was operated in the positive linear mode with an accelerating voltage of 25000V. Proteins masses were screened from 5000 – 45000 Da with a low mass gate of 5000. Data were acquired with 500 shots/ spectrum and summed up for the final analysis.

2.7. Enrichment of photolabeled MMP-12 CD on monomeric avidin beads

High affinity biotin-binding sites (oligomeric avidin groups) of monomeric avidin (Pierce) were first blocked with a biotin-containing buffer. To this purpose, 50 µL of beads were washed twice with 1 mL of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl buffer for 10 min before blocking oligomeric avidin binding sites by incubation for 1h with 500 µL of the above buffer containing 2 mM biotin. Biotin was removed from the lower affinity monomeric binding sites by elution (2 washes for 10 min each) with 1 mL of regeneration buffer (0.1 M glycine in 50 mM Tris-HCl, pH 2.8, 150 mM NaCl) and two final washes (10 min each) with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl according to the manufacturer's instructions. All steps were performed at room temperature.

For enrichment of the labeled protein, 10µL of monomeric avidin beads diluted to 100 µL with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 2 µg of the labeled protein were incubated for 1h at room temperature. After 4 washes with 300 µL of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl (for 10 min each at room temperature) proteins were eluted with 3-times 10 µL of 8 M guanidinium-HCl, pH 1.8 at 65°C for 2 min.

2.8. SDS PAGE analysis

Discontinuous, reducing SDS-PAGE was performed according on 1 mm thick gels in a Mini protean III cell assembly (BioRad). Experimental conditions were as follows: stacking gel (1 cm), 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.2% (v/v) TEMED, 125 mM Tris-HCl, pH 6.8; separating gel (6 cm), 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.04% (v/v) TEMED, 375 mM Tris-HCl pH 8.8; running buffer, 25 mM Tris base, 200 mM glycine, pH 8.3, 0.1% (w/v) SDS. One volume of loading buffer (10% SDS (w/v), 10 mM DTT, 20% glycerol (v/v), 200 mM Tris-Hcl pH 6.8, 0.05% bromophenol blue (w/v)) was added to four volumes of samples. Samples were heated for 5 min at 95°C and loaded on the gel directly after heating. Running conditions: 10 min at ~120 V (stacking) and 50 min at ~160 V until migration of the bromophenol blue to the end of the gel. After electrophoresis, gels were stained according to Yan *et al*. [19].

2.9. Tryptic digestion

Tryptic digestion was performed on labeled and non-labeled protein after exchanging the labeling medium against 50 mM of ammonium bicarbonate by ultrafiltration at 7 kDa (Zeba Spin

Desalting Columns (Pierce, cat. n° 89882)) according to the manufacturer's instructions. Alternatively, proteins were denatured by methanol-chloroform precipitation (see 2.4. Labeling of MMP-12 CD). Samples were then digested for 4 hours with trypsin (1:30 enzyme to protein ratio) in a final volume of 10 µL of 50 mM of ammonium bicarbonate. Tryptic digest were mixed with the loading buffer for Tricine-SDS-PAGE analysis.

2.10. Tricine-SDS PAGE and Western blotting

Electrophoresis was performed according the protocol of Schägger (18% Tricine-SDS-PAGE, 1 mm thickness containing a 10% stacking gel (1 cm)) [20]. The proteins were transferred to an Immun-Blot PVDF (polyvinylidene fluoride) membrane by wet blotting in a mini Trans-blot cell at 350 mA for 60 min in 25 mM Tris, 190 mM glycine with 20% v/v methanol (BioRad). Membranes were blocked overnight at 4°C in TBST (25 mM Tris, pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20), supplemented with 5% w/v non-fat dried milk (Protifar Plus, Nutricia, Zoetermeer, The Netherlands) and incubated for 1 h in a 1:1500 dilution of streptavidin-alkaline phosphatase (0.67 µg/mL) in TBST (25 mM Tris buffer pH 7.5 containing 150 mM NaCl, 0.05% v/v Tween-20) supplemented with 1% non-fat dried milk. Biotinylated proteins were visualized by staining with a BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium chloride) tablet (Sigma Aldrich) in 10 mL of ultrapure water.

2.11. Fluorescent measurements

Activity assays were performed in 96-well plates (Costar-white) on a Fluostar Optima plate reader (BMG, Labtech) with 100 µL of MMP assay buffer. The enzyme activity (MMP-12 CD or ^{15N}MMP-12 CD spiked at 10 ng/100 µL) was followed by monitoring (for 15 min) conversion of the fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 (BACHEM) spiked at 2µM (λex, λem=327, 420 nm).

3. Results and discussion

3.1 Expression, purification, and refolding of MMP-12 CD

The determination of the photoaffinity labeling site as well as characterizing the ratio of the enzyme:probe requires high amount of MMPs. MMP-12 CD was thus purified and expressed (catalytic domain G_{106} to N_{268} plus the initiator M at the N-Ter) in *E. coli* according to the publication Parkar *et al.*[17].

MMP-12 CD was expressed mainly in inclusion bodies and purified by size exclusion chromatography (SEC) before being refolded in two steps: dialysis followed by cation exchange chromatography (see 2.3. Expression of recombinant MMP-12 CD).

Figure 2 gives an overview of the purification and refolding procedure as analyzed by SDS-PAGE. MMP-12 CD was the major protein in the inclusion bodies and the only detected protein after

the SEC step (**Figure 2**, lane SEC1-2). The analysis of the first refolding step (dialysis) by SDS-PAGE shows some autodegradation products as distinct bands below the major MMP-12 CD band (**Figure 2**, lane AD). These degradation products are removed by the second refolding step which is based on strong cation-exchange chromatography. The final product shows a single protein band of 15-20 kDa upon SDS-PAGE (**Figure 2,** lanes SCX1-3).

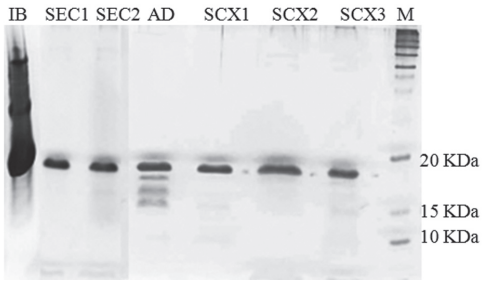

Figure 2. SDS PAGE analysis of the purification and refolding of MMP-12 CD (106-268 plus one extra N-terminal M) following the procedure of Parkar *et al*. [17]. MMP-12 CD was expressed in *E. coli* and accumulated in inclusion bodies. The protein was solubilized and purified by size exclusion chromatography and refolded by dialysis and strong cation exchange chromatography.
IB: inclusion bodies; SEC1-SEC2 : fraction after purification by size exclusion chromatography ; AD: fraction partially refolded after dialysis; SCX1-SCX3: fraction refolded and purified by strong cation-exchange chromatography (final product); M: marker.

Purification and refolding of MMP-12 CD was also followed by an enzyme activity assay using a pro-fluorescent peptide substrate showing that activity of the final product was similar to a ^{15}N-labelled MMP-12 CD standard (gift from AstraZeneca). Two liters of culture resulted in about 0.8 mg of purified MMP-12 CD (see **Table 1**).

Sample	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Total protein (%)
Inclusion bodies solubilized in 8 M guanidine–M HCl	10	62.5	625	100
6 M guanidine–HCl pool after gel filtration	100	1.29	129	20.6
6 M guanidine–HCl (an aliquot of 2.1mg protein was used for refolding)	1.2	1.75	2.1	20.6
3 M urea dialysate	20	0.1	2	19.6
SP–Sepharose pool (final product)	4	0.19	0.76	7.1

Table 1. Purification table of MMP-12 CD from 2 liters of *E. coli* culture.

MALDI-TOF-MS analysis showed that the measured molecular mass of the final product (18215.3 Da) is in close agreement with the expected mass based on the cloned sequence with an additional N-terminal Met (18208.3 Da) (**Figure 3**).

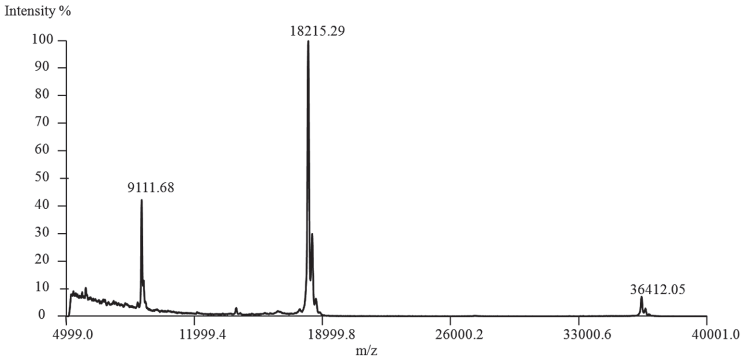

Figure 3. Analysis of MMP-12 CD (106-268 plus a Methionine at the N-terminal extremity) by MALDI-TOF after the second refolding step by strong SCX. The molecular weight 18215.3 Da is in close relation with the calculated molecular weight of 18208.3 Da.
The observed peaks of 9111.7 Da and 18215.3 a correspond to the monomer of MMP-12 CD (doubly and singly-charged respectively). The dimer (singly-charged) of MMP-12 is also observed with a molecular weight of 36412 Da.

3.2. Stoichiometry of the labeling

An ideal activity-based probe should result in covalent and active-site directed labeling. A previous study described two different probes, based on high-affinity hydroxamate based metalloprotease inhibitors with a photoreactive trifluoromethyldiazirine group and a biotin moiety for detection for affinity-enrichment of the targeted protein [16]. One of these two probes has the photoreactive group directed at the S2' pocket (structure not shown) while the other targets the S1' pocket in the active site (see **Figure 1,** Probes 1 and 2). Both probes label ADAMs (A Disintegrin And Metalloproteases) and MMPs in an activity-dependent manner but targeting the S1' pocket leads to broad-spectrum labeling of MMPs while the probe addressing the S2' pocket labels mainly MMP-9 and MMP-10 of the panel of tested enzymes. Labeling is covalent and site-specific as demonstrated by competition experiments with a probe lacking the biotin moiety and through competition with inhibitors of the Tissue Inhibitor of MetalloProtease (TIMP) family for both probes.

To study the interaction between probe 2 and MMP-12 CD we determined the stoichiometry of labeling of the ^{15}N-MMP-12 CD by LC-MS using the non-biotinylated, active-site-directed probe targeting the S1' pocket (Probe 2, **Figure 1B**). LC-MS analysis confirmed the expected 1:1 stoichiometry, since the measured mass difference of 461.8 Da due to photoaffinity labeling with Probe 2 corresponded closely to the calculated value of 463.4 Da (see **Figure 4**). We did not observe any further labeling indicating the absence of non-specific protein labeling. This result lends further support to the specific, activity-dependent labeling of the active site of MMP-12 CD.

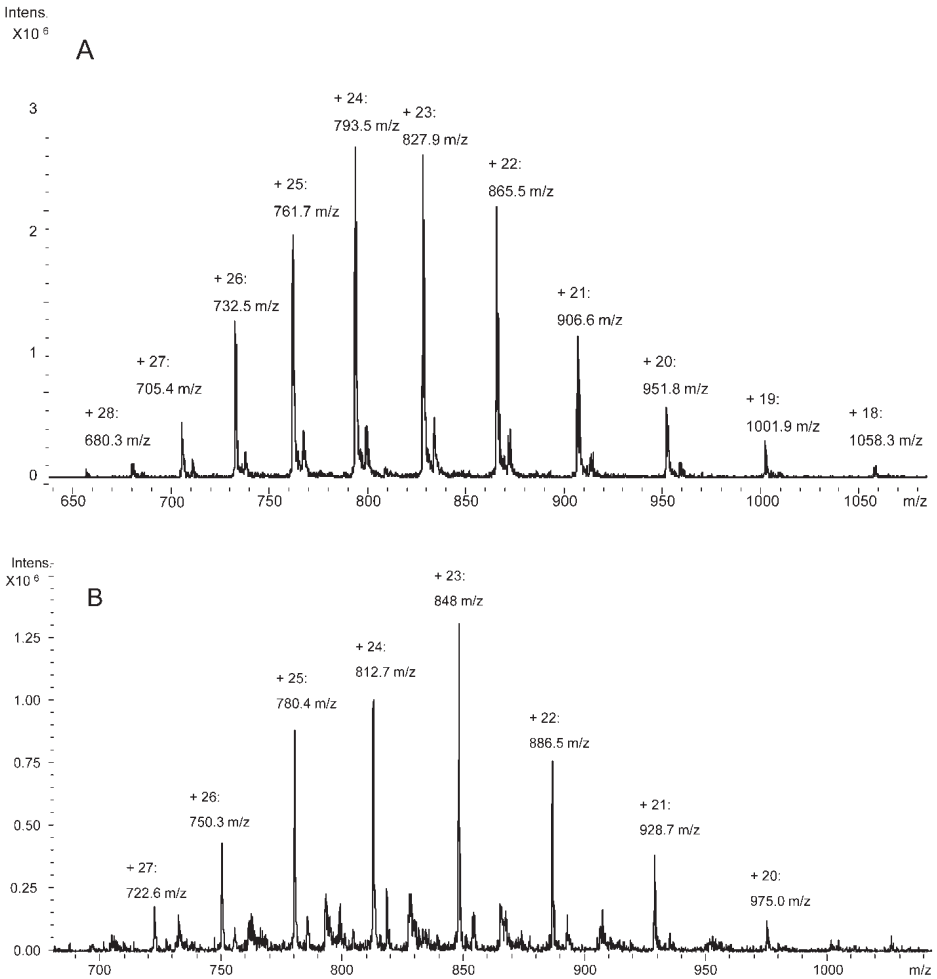

Figure 4. Electrospray ionization mass spectra of non-labeled recombinant MMP-12 CD (A) and MMP-12 CD photoaffinity-labeled with Probe 2 (B) after LC-MS analysis. Mass spectra were combined over retention time windows corresponding to the non-labeled (42.1 – 45.7 min) and the labeled proteins (45.0 - 46.6 min). Measured molecular masses based on ^{15}N-MMP-12 CD were: 19018.9 Da (non-labeled) and 19480.7 Da (Probe 2-labeled). Measured mass difference: 461.8 Da; calculated mass difference: 463.4 Da.

3.3. Tryptic digestion of labeled MMP-12 CD

Determining the photolabeling site (amino-acid(s) involved in the covalent bond with the synthetic probe) requires enzymatic digestion (e.g. with trypsin) of the labeled enzyme before characterizing the modified peptides. Efficient tryptic digestion is thus a prerequisite for identifying labeled-peptides. Digestion efficiency was evaluated by analyzing labeled and non-labeled MMP-12 CD by Tricine-SDS-PAGE.

Tryptic digestion of the labeled/inhibited and control proteins was done after chloroform-methanol precipitation. Chloroform-methanol precipitation allows protein denaturation, protein concentration as well as desalting. Tryptic digestion efficiency of the control protein (see **Figure 5b** lane CD) was more complete than of the labeled/inhibited protein (**Figure 5b** lane LD). The reason for this discrepancy is currently unclear.

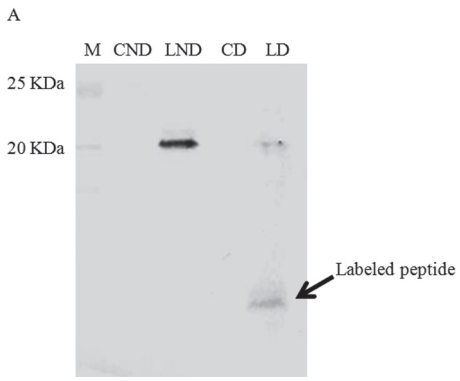

Figure 5A. Western blot analysis (developed with streptavidin alkaline phosphatase) after protein separation by 18% Tricine-SDS PAGE. Tryptic digestion of MMP-12 CD that was labeled with Probe 1 (which includes a biotin moiety (see **Figure 1B**)) and non-labeled MMP-12 CD. Proteins were denatured via methanol-chloroform precipitation prior to trypsin digestion.

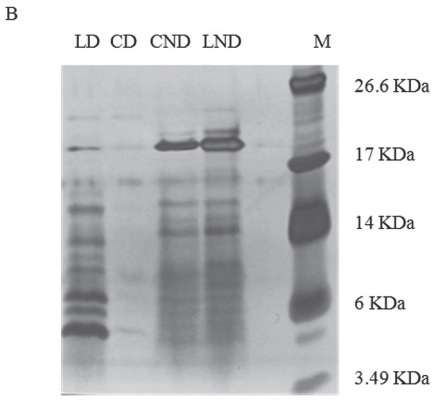

Figure 5B. Tricine-SDS PAGE of MMP-12 CD labeled with Probe 1 (which includes a biotin moiety (see **Figure 1B**)) and non-labeled MMP-12 CD. Proteins were denatured via methanol-chlororform precipitation prior to trypsin digestion.
M: Molecular weight marker; LD: labeled trypsinized MMP-12 CD; CD: control trypsinized MMP-12 CD; LND: labeled non-trypsinized MMP-12 CD; CND: control non-trypsinized MMP-12 CD**.**

3.4. Enrichment of labeled MMP-12 CD on monomeric avidin beads

Previous work on labeling enzymes with ABPs was based on including a biotin tag in the probe and enriching labeled proteins using immobilized streptavidin. While this principle allows considerable enrichment of the target enzyme from complex biological samples, it has proven difficult to recover the bound protein short of using rather harsh conditions (e.g. boiling under reducing conditions with Laemmli buffer prior to gel electrophoretic analysis), which are often incompatible with subsequent mass spectrometric analysis as needed to identify the enriched protein(s).

To facilitate protein recovery, labeled-protein enrichment was performed with immobilized monomeric avidin beads. **Figure 6** shows enrichment of Probe 1-labeled MMP-12 CD followed by anti-biotin Western blotting of the obtained fractions. Results of the **Figure 6** indicate high recovery of the labeled protein after elution with 8M guanidinium-HCl, pH 1.8. Guanidinium-HCl can be easily removed by methanol-chloroform precipitation prior to LC-MS analysis or tryptic digestion.

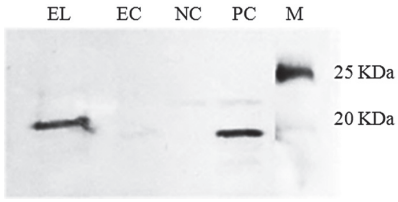

Figure 6. Western blot analysis of recombinant MMP-12 CD photoaffinity labeled with Probe 1, which includes a biotin moiety (see **Figure 1A**). The blot was developed with streptavidin alkaline phosphatase after separation by 18% Tricine-SDS PAGE. M: Molecular weight marker; PC: labeled MMP-12 CD prior to affinity enrichment; NC: non-labeled MMP-12 CD; EL: elution fraction of labeled MMP-12 CD from the monomeric avidin column; EC elution fraction of non-labeled MMP-12 CD from the monomeric avidin column**.**

4. Conclusions

Succinyl hydroxamate-based metalloprotease inhibitors containing the photoreactive trifluoromethylphenyldiazirine group were designed and synthesized to label MMPs and ADAMs [16]. The present study extends this work by showing that MMP-12 CD is labeled at a 1:1 stoichiometry. Incorporating a biotin moiety into the photoreactive probe allows enriching the labeled protein on monomeric avidin beads opening. We are currently improving the design of photoactivatable probes in order to reach significantly higher labeling efficiencies (currently at the level of 1-5%) on a determine the labeling site. Further improvements in broad spectrum labeling on a family-wide scale to render this approach suitable for in depth biological studies into the roles of active MMPs and ADAMs in disease development.

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Chapter 3

Quantification of Matrix Metalloprotease-9 in Bronchoalveolar Lavage Fluid by Selected Reaction Monitoring with Microfluidics nano-Liquid-Chromatography

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Abstract

Quantitative protein analysis by liquid chromatography – tandem mass spectrometry (LC-MS/MS) in the selected reaction monitoring (SRM) mode was used to quantify matrix metalloprotease – 9 (MMP-9) in bronchoalveolar lavage fluid (BALF) from patients having undergone lung transplantation. We developed an SRM assay for microfluidics-based nanoLC-MS/MS on a triple quadrupole mass spectrometer based on two signature peptides. Samples were prepared by chloroform-methanol precipitation followed by trypsin digestion in the presence of stable-isotope-labeled internal peptide standards. The method allows accurate quantification of MMP-9 in BALF with an LLOQ of 2.9 ng/mL and an LLOD of 0.25 ng/mL without the use of extensive fractionation or antibodies. A study of 10 BALF samples (5 with and 5 without acute rejection of the transplant) did not show a correlation between the level of MMP-9 and acute rejection.

1. Introduction

Clinical validation of potential biomarkers requires quantitative analyses. Most current quantitative analyses rely on Enzyme-Linked ImmunoSorbent Assays (ELISA). This technique is one of the most sensitive approaches (pg/mL range) to measure proteins in complex biological samples. To develop a specific and sensitive ELISA in the most common 'sandwich' format, two antibodies recognizing different epitopes with high specificity and affinity in the same protein are required. The need for two 'orthogonal' high-affinity antibodies may render assay development costly and time consuming. It is furthermore uncertain whether the antibodies recognize the target protein specifically, since the readout of the assay is indirect (color, fluorescence, chemiluminescence).

In recent years, there has been increasing interest in the use of liquid chromatography – tandem mass spectrometry (LC-MS/MS) in the selected reaction monitoring (SRM) mode for protein quantification [1-8]. The main advantage of the SRM-based protein assay is the facile multiplexing due to the capacity of modern triple quadrupole mass spectrometers to monitor a range of peptides in a single chromatographic run using scheduled SRM with the respect of the specificity. However, the main drawback of this technique, compared to immunoassays, is its lower sensitivity. SRM protein quantification in plasma, without immunodepletion and/or extensive fractionation steps, allows protein detection at the low µg/mL level [9, 10]. To reach the low ng/mL range in serum, reducing sample complexity is mandatory and achieved by applying immunoaffinity enrichment with immobilized antibodies [11-19] and/or prefractionation [20, 21] which also reduces ion suppression. Prefractionation or enrichment allows further to introduce higher amounts of the target protein into the analytical system making better use of the generally limited binding capacity of the LC column, which remains one of the sensitivity-limiting factors in protein analysis by LC-MS/MS.

SRM assays have been primarily developed for protein analysis in plasma and serum, which are easily obtained in sufficient quantity during routine medical visits. Bronchoalveolar Lavage Fluid (BALF) is, however, a more suitable sample when studying pulmonary disease, since BALF gives a representative picture of processes occurring in the airways. The characteristics of this matrix for targeted protein analysis have, however, been much less studied.

Transplantation remains the ultimate therapy for patients with end-stage pulmonary disease [22, 23] but may be accompanied by early complications such as acute rejection of the transplanted lung. Matrix metalloproteases (MMPs), belonging to the family of zinc-dependent endopeptidases, are involved in a wide range of biological processes related to extracellular matrix (ECM) remodeling and tissue repair [24, 25]. MMPs are tightly regulated *in vivo* and any deregulation may lead to broad pathological processes. Notably MMP-9 has been shown to be upregulated during chronic inflammation (e.g. during allergic asthma) [25, 26]. To further assess the role of MMP-9 in pulmonary disease and notably in the rejection of lung transplants, we established a human MMP-9-specific SRM assay.

This assay requires generic sample preparation consisting of chloroform-methanol precipitation and trypsin digestion. The LC-MS/MS assay uses a fully automated microfluidics-based nanoLC-

MS/MS system coupled on-line to a triple quadrupole mass spectrometer and allows quantifying MMP-9 at the low ng/mL level without the use of immunoaffinity enrichment and/or extensive prefractionation.

2. Materials and Methods

2.1. Reagents and chemicals

Ammonium bicarbonate, dimethyl sulfoxide, chloroform, dithiothreitol, formic acid, and iodoacetamide were purchased from Sigma Aldrich. Formic acid and methanol were ordered to Fluka. Trypsin (sequencing grade, cat. n° V5111) was from Promega, NaCl was obtained from Merck and acetonitrile from Biosolve. Ultrapure water (resistivity of 18.2 MΩ·cm) was obtained from a Sartorius Stedim water purification system (model 611 VF). The following stable isotope-labeled internal standard peptides were obtained from JPT Peptide Technologies (http://www.jpt.com): AVIDDAFAXSAZ; AFALWSAVTPLTFTXSAZ (X= $Arg^{13}C_6$, 97-99%; $^{15}N_4$, 97-99%, Z=JPT-Tag, which is removed during tryptic digestion).

2.2. Human recombinant MMP-9 catalytic domain (rec. MMP-9 CD)

0.1 mg/mL in 50 mM Tris, 10 mM $CaCl_2$, 20 µM $ZnCl_2$, 150 mM NaCl, 0.02% Brij, pH 7.5, without the fibronectin type II-like repeats (residues 107-216 fused to residues 391-443 with an additional Met-Gly dipeptide at the N terminus**)** was a kind gift from AstraZeneca and expressed according to the protocol of Shipley *et al.*[27].

2.3. BALF

BALF was obtained from patients having undergone lung transplantation for COPD (8 patients), cystic fibrosis (1 patient) or idiopathic pulmonary fibrosis (1 patient) (**Table 1**). BALF was collected as described elsewhere [28] and stored at -80 ºC. The procedures followed were in accordance with ethical and national local guidelines.

Sample Number	Gender and age (years)	Days between transplantation and BALF sampling	Disease	Total cells $\times 10^6$	Lymp (%)	Neut (%)	Eo(%)	IL6 (pg/mL)	IL8 (pg/mL)	Total protein concentration µg/mL
Acute rejection of the lung transplant										
406	M 57	13	IPF	0.14	5	2	2	2	7	49.0
404	F 23	71	CF	0.11	4	1	0	2	79	92.3
386	M 63	22	COPD	0.27	2	0	0	2	5	28.1
389	M 49	40	COPD	0.12	6	4	0	3	720	88.5
392	F 63	18	COPD	0.50	7	1	0	31	28	531.3
Without acute rejection of the lung transplant										
383	M 54	107	COPD	0.20	2	4	0	3	13	80.6
394	F 55	31	COPD	0.43	4	1	0	3	42	93.5
395	M 59	36	COPD	0.07	3	6	0	10	401	123.9
390	M 61	106	COPD	0.09	2	9	0	49	1127	185.1
396	M 61	58	COPD	0.33	4	6	0	12	121	124.1

Table 1. Clinical characteristics of BALF samples from lung transplantation patients with or without acute rejection of the transplant. The total protein concentration was determined in duplicate measurement with the µBCA kit (see Materials and Methods). Lymp: Lymphocytes; Neut: Neutrophiles; Eo: Eosinophiles. IPF: Idiopathic Pulmonary Fibrosis. CF: Cystic Fibrosis. COPD: Chronic Obstructive Pulmonary Disease

2.4. IL-6 and IL-8 Measurements

IL-6 and IL-8 were measured in BALF by a chemiluminescent immunoassay (Immulite IL-6 and Immulite IL-8, Siemens Healthcare Diagnostics Products LTD, Gwynedd, UK) performed on an Immulite 1000 system.

2.5. Determination of total protein concentration

BALF was diluted 3-fold with PBS (Phosphate Buffer Saline; 140 mM NaCl, 9 mM Na_2HPO_4, 1.3 mM $Na_2H_2PO_4$, pH 7.4) to determine the total protein concentration with the microBCA assay according to the manufacturer's instructions (Pierce). Absorbance was measured with a Fluostar Optima plate reader (BMG, Labtech) at 580 nm.

2.6. Sample preparation

BALF (10 µg of total protein) or rec. MMP-9 CD (2.5 µg), was diluted to a final volume of 130 µL in PBS, reduced with 4 mM dithiothreitol (15 min at 56 °C) and then alkylated with 12 mM iodoacetamide (20 min at room temperature in the dark). Proteins were precipitated prior to tryptic digestion with chloroform-methanol according to Wessel and Flugge [29]. Briefly, samples were mixed with 600 µL methanol, 150 µL chloroform and 450 µL water by vortexing and centrifuged at 13600 g for 5 min. The pellet was washed with 450 µL of methanol and centrifuged as above. The final pellet was dried under the fume hood, suspended in 20 µL of 50 mM ammonium bicarbonate buffer and digested for 5 h at 37 °C (ratio trypsin/protein: 1/30, w/w).

2.7. Selection of signature peptides and SRM optimization

To assess the digestion efficiency for rec. MMP-9 CD and to select peptides for quantification, the triple quadrupole mass spectrometer was operated in full scan and product ion scan mode with the fragmentor (FR) set at 135 volt, a dwell time of 150 ms and collision energies (CE) of 4, 12, 20, 30 or 50 volt. The most promising transitions were further optimized with steps of 4 volts for CE and 10 volts for FR. This optimization was done using the Agilent MassHunter Optimizer Automated MS Method Development Software (version B.02.01) injecting 1 pmol of trypsin-digested rec. MMP-9 CD.

2.8. Optimization of MS-MS selectivity and sensitivity

To improve sensitivity, resolution of the first and third quadrupole mass analyzer were set to ±0.35 unit (u), ±-0.6 u or ±1.25 u (full width at half height), respectively. To determine the optimal resolution of the third quadrupole, the first quadrupole was kept at±0.35 u. To determine the optimal resolution of the first quadrupole, the third quadrupole was kept at ±1.25 u. Sensitivity for the selected SRM transitions was assessed by determining the peak area of the quantifier ion of

the targeted peptide after injecting 14 fmol of rec. MMP-9 CD in BALF after digestion with trypsin (8 µg total protein; samples were spiked to 1.4 ng/mL of rec. MMP-9 CD corresponding to a final concentration of 12.96 ng/mL tryptic rec. MMP-9 CD digest in BALF that was injected). Selectivity of the measurements was evaluated by comparing the ratio of the transitions in the presence and absence of biological matrix at each of the resolution settings to assess whether peptides related to trypsin-digested BALF proteins interfered with the target peptides.

2.9. LC-MS/MS

Samples were analyzed by nanoLC-MS/MS using a microfluidics (chip-cube) interface (Agilent, cat. n° G4240A) on a C-18 chip (Agilent, custom-made) with a 500 nL enrichment column (Zorbax 300 SB C-18, 5 µm) and a 75 µm × 150 mm separation column packed with the same chromatographic material. The interface contained a nanoelectrospray tip (2 mm length with conical shape: 100 µm OD × 6 µm ID) that was coupled on-line to a triple quadrupole mass spectrometer (Agilent, G6410B). Tryptic digests (4 µg total protein), dissolved in 5% formic acid (FA) / 5% dimethylsulfoxide (DMSO), were loaded on the trap column in 0.1 % aq. FA, 3% acetonitrile at 2.5 µL/min via the autosampler (Agilent, cat. no. G1377A) equipped with an injection loop of 40 µL (Agilent, cat. no. G1377A) and a thermostated cooler (Agilent, cat. no. G1330B) maintaining the samples in the autosampler at 4° C. The interface was connected to an Agilent 1200 series HPLC system containing the following modules: nanopump (Agilent, cat. no. G2226A), capillary pump (Agilent, cat. no. G1376A) and solvent degasser (Agilent, cat. no. G1379B). The samples were enriched in the forward flushing mode (in 0.1% aq. FA, 3% acetonitrile, and flushed with an extra 4 µL volume using the capillary pump at 2.5 µL/min) after which the trapping column was switched in-line with the analytical column. Peptides were eluted with eluents A (0.1% FA in water) and B (0.1% FA in acetonitrile) at a flow rate of 0.25 µL/min using the following gradient program: 3% to 10% eluent B at 2%/min , 10% to 35% eluent B at 0.5%/min, 35 to 60% eluent B at 2 %/min followed by 60% to 95% eluent B at 11.6%/min. Eluent B (95%) was maintained for 3 min before returning to the starting conditions over 10 min. The column was equilibrated at the starting conditions for 10 min before starting the next injection. Carry-over was avoided by washing the needle for 10 s with 0.1% FA in 20 % methanol.

Calibrants and samples were analyzed with wide resolution (±-0.6 u at half height) for the first quadrupole and widest resolution (±-1.25 u at half height) for the third quadrupole. Each transition was monitored with a dwell time of 150 ms in segmented SRM mode as follows: 0 - 39 min; target peptide 489.3→404.2/579.3/694.3/807.4 FR 210, CE 8/22/13/12; internal standard: 494.3→409.2/589.3/704.3/817.4, FR 210, CE 8/22/13/12; 39 - 92 min; target peptide 841→219.1/290.1/1092.6, FR 220, CE 41/49/33; internal standard: 846→219.1/290.1/1102.6 FR 220 CE 41/49/33.

2.10. Calibration curves without and with biological matrix

A tryptic digest of rec. MMP-9 CD was diluted in 5% FA / 5% DMSO to establish a calibration curve at the following concentrations (52 ng/mL, 26 ng/mL, 13 ng/mL, 8.64 ng/mL, 5.2 ng/mL, 0 ng/mL) using the optimized SRM method. Prior to analyzing BALF samples from lung transplantation patients, a calibration curve was established in a pool of 13 BALF samples (8 lung transplantation patients and 5 patients with other pulmonary pathologies such as COPD or sarcoidosis) spiked with rec. MMP-9 CD as follows: 0 ng/mL, 0.15 ng/mL, 0.5 ng/mL, 2.88 ng/mL, 9.6 ng/mL, 11.56 ng/mL, 28.91 ng/mL and 57.8 ng/mL, which corresponds to a concentration of the tryptic digests prior to SRM analysis of: 0 ng/mL, 0.28 ng/mL, 0.94 ng/mL, 5.41 ng/mL, 18.04 ng/mL, 21.73 ng/mL, 54.35 ng/mL and 108.6 ng/mL. The samples for each calibrant point were prepared separately in duplicate and analyzed once by LC-MS/MS. Stable-isotope-labeled internal peptide standards (AVIDDAFAXSAZ; AFALWSAVTPLTFTXSAZ) were added prior to tryptic digestion to a final amount of 10 fmol per sample of which 4 fmol were injected.

2.11. Repeatability of sample preparation and protein recovery

BALF samples from three different patients were spiked with 1.4 ng/mL of rec. MMP-9 CD corresponding to a final concentration of 12.96 ng/mL tryptic digest and measured by SRM as described above to assess the repeatability of sample preparation. Protein recovery was assessed in triplicate by measuring the total protein concentration after dissolving the protein pellet in 2 % aq. sodium dodecyl sulfate (SDS) after chloroform-methanol precipitation.

2.12. Analysis of MMP-9 in BALF from lung transplantation patients

BALF samples from 10 patients (**Table 1** for details) were randomized (MATLAB random permutation test) and prepared in duplicate (see **Figure 1** for a general overview). Prior to and after LC-MS/MS analysis of the BALF samples (a volume of 17.7 µL was injected for all samples and all calibration points), calibration curves were established to assure consistent system performance .System performance and stability were further followed by injecting 7 control samples (trypsin-digested rec. MMP-9 CD) in regular intervals.

2.13. Estimation of LOQ and data processing for semi-quantification of MMP-9

LOQ (Limit of Quantification) corresponding to 3 and 10 times the Signal to Noise Ratio (SNR) respectively were estimated manually by selecting a baseline region of 0.5 - 1 min following the peptide peak for noise estimation. Data were processed in an automated manner with the quantitative software of Agilent (version B.03.02) and manually verified with the qualitative software (version B.01.03). The relative response uncertainty was set to± 25 for the ratio of the monitored transitions and Gaussian smoothing (15 and 5 points for function and Gaussian width respectively) was applied. Peaks were integrated automatically with the algorithm of the Agilent software.

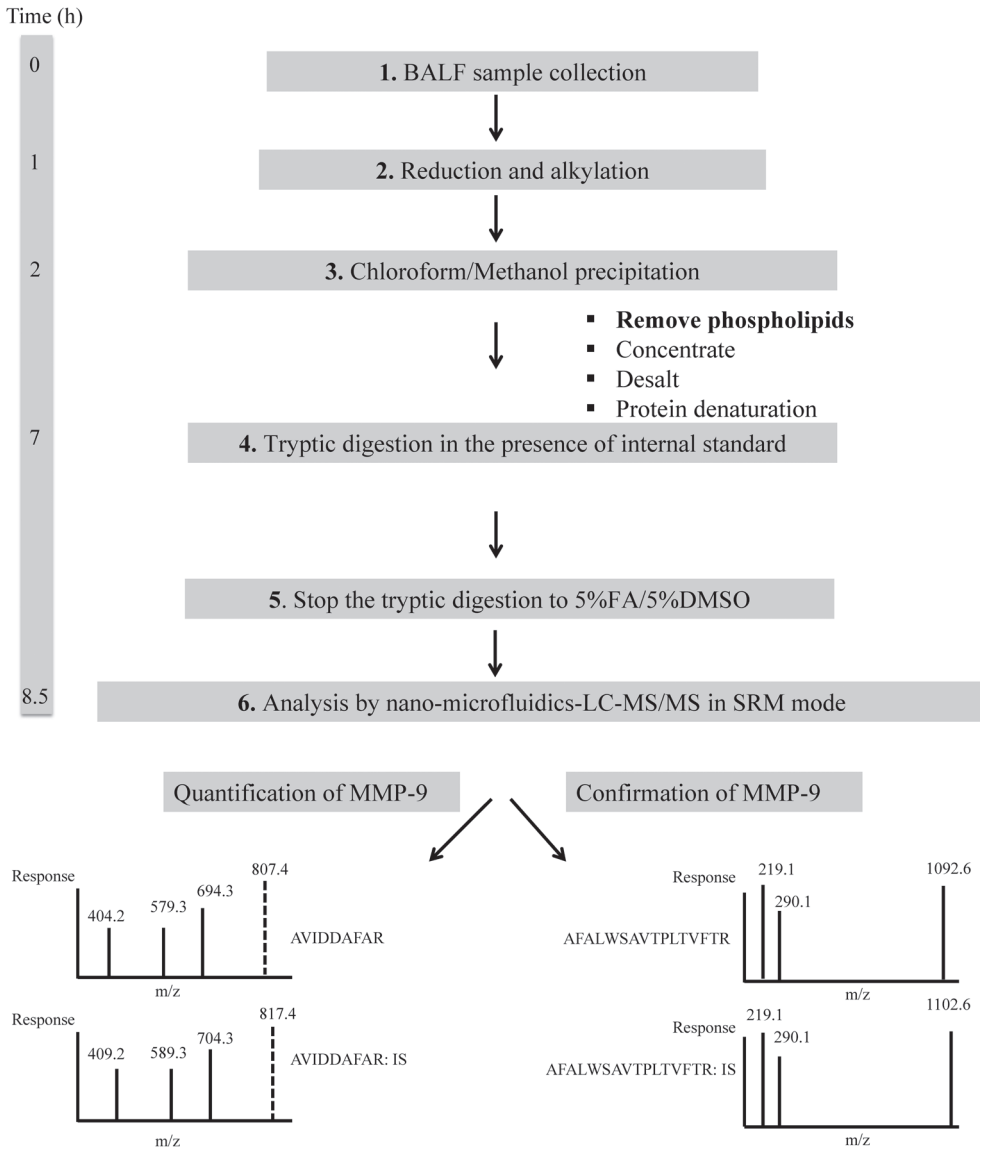

Figure 1. Schematic overview of the overall sample preparation procedure of BALF samples for human MMP-9 analysis by SRM. Aliquots of BALF were reduced and alkylated prior to chloroform-methanol precipitation. Precipitation allows to desalt and concentrate the samples, denature proteins prior to tryptsin digestion, and remove the abundant phospholipids.
To develop a high-sensitivity SRM assay for human MMP-9, two peptides were targeted. AVIDDAFAR was used for quantification due to its good response and AFALWSAVTPLTFR was followed to provide confirmatory evidence. Three or 4 transitions per peptide were followed (the transition that is represented as a dashed line is used for quantification).
The time axis on the left gives an estimate of the required time, showing that the entire procedure can be completed in one day. It is possible to prepare multiple samples in parallel for SRM analysis.

2.14. Application of the developed assay to another biological matrix: Epithelial Lining Fluid (ELF) analysis

A new sampling approach has been described for collecting pulmonary fluids [30, 31]. Such a procedure has the advantage to give less diluted samples than BALF. ELF sample preparation for SRM analysis was similar than for BALF. The samples (corresponding to an original amount of 4 µg of total protein) were first diluted to a final volume of 130 µL in PBS buffer, before being reduced for 15 min at 56 ºC with 3 mM of dithiothreitol and then alkylated for 20 min at room temperature with 9 mM iodoacetamide in the dark. Protein concentration and denaturation were achieved by methanol/chloroform precipitation prior to trypsin digestion.

Biological matrix to perform the calibration curve was a pool of 6 ELF samples (2 samples from COPD (Chronic Obstructive Pulmonary Disease) patients, 1 control sample and 2 lung transplant samples). Five calibrant points were measured: 1.9 ng/mL, 5.3 ng/mL, 10.7 ng/mL, 16 ng/mL and 33.3 ng/mL for MMP9 and MMP12 spiked into this biological matrix.

Four samples were analyzed separately: 2 samples from COPD (Chronic Obstructive Pulmonary Disease) patients, 1 control sample and 1 pool of 2 samples from lung transplantation samples.

3. Results

3.1. Choice of signature peptides and SRM optimization

The choice of signature peptides is a crucial step to obtain a sensitive, specific, and reproducible SRM assay. In order to select the most appropriate peptides, the catalytic domain of rec. MMP-9 (MMP-9 CD) was digested with trypsin and the digest analyzed by LC-MS on a triple quadrupole mass spectrometer (**Figure 2**).

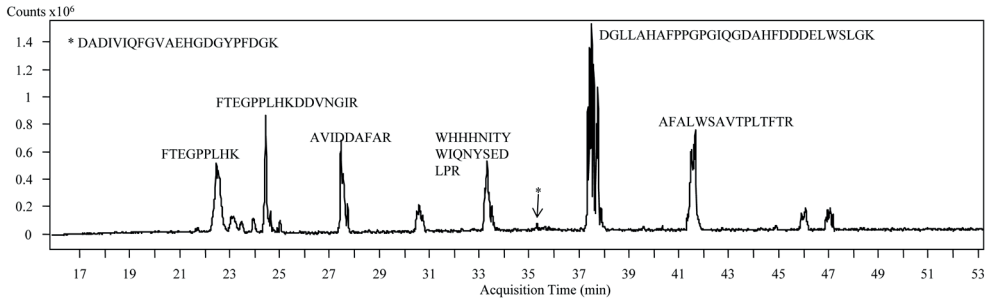

Figure 2. Base peak chromatogram of a tryptic digest of recombinant MMP-9 (catalytic domain). 1 pmol was analyzed by microfluidics-based nanoLC-MS on a triple quadrupole mass spectrometer (scan range: 350 -2000 m/z) in order to select the most suitable signature peptide for developing the SRM assay.

Peptides of excessive length, containing instable amino acids such as M or C or missed cleavage sites were discarded, since they may lead to poorly reproducible SRM assays. The remaining peptides WHHHNITYWIQNYSEDLPR, AVIDDAFAR, and AFALWSAVTPLTFTR were subjected to a sequence homology search using the BLASTP network service against the UniProt Knowledgebase (Release 2011_07, 28-Jun-11) database consisting of the UniProtKB/Swiss-Prot database (530264 entries) and the UniProtKB/TrEMBL (16014672 entries) with specification "*Homo sapiens*" to assure that they do not occur in any other human protein. These three peptides were further analyzed with respect to their suitability for developing selective, high-sensitivity, quantitative SRM assays. While peptide WHHHNITYWIQNYSEDLPR showed promising results with a pure rec. MMP-9 CD digest, it was no longer detectable when rec. MMP-9 CD was added to BALF. This peptide was thus not followed up. Product ion scans of the other two peptides at increasing collision energies resulted in suitable MS/MS spectra for AVIDDAFAR and somewhat less intense fragment ions for AFALWSAVTPLTFTR (**Figure 3**).

Peptide sequence	Parent ion (m/z)	FR (V)	Product ion (m/z)	CE (V)
AVIDDAFAR	489.3 (+2)	210	807.4 (y7; +1)	12
			694.3 (y6;+1)	13
			579.3 (y5;+1)	22
			404.2 (y7;+2)	8
AVIDDAFA**R**	494.3 (+2)		817.4 (y7; +1)	12
			704.3 (y6;+1)	13
			589.3 (y5;+1)	22
			409.2 (y7;+2)	8
AFALWSAVTPLTFTR	841 (+2)	220	1092.6 (y10;+1)	33
			219.1 (b2;+1)	41
			290.1 (b3;+1)	49
AFALWSAVTPLTFT**R**	846 (+2)		1102.6 (y10;+1)	33
			219.1 (b2;+1)	41
			290.1 (b3;+1)	49

Table 2. Optimized parameters for MMP-9-specific SRM assays based on peptides AVIDDAFAR and AFALWSAVTPLTFR monitoring 4 or 3 product ions, respectively. Peptides labeled with ^{15}N and ^{13}C at the C-terminal R (shown in bold-underlined) were chosen as internal standards. FR: fragmentor voltage. CE: collision energy.

Optimized SRM conditions for AVIDDAFAR and AFALWSAVTPLTFTR are given in **Table 2**. Peptides labeled with ^{15}N and ^{13}C at the C-terminal R residue were used as internal standards and subjected to collision-induced dissociation under identical conditions (**Table 2**).

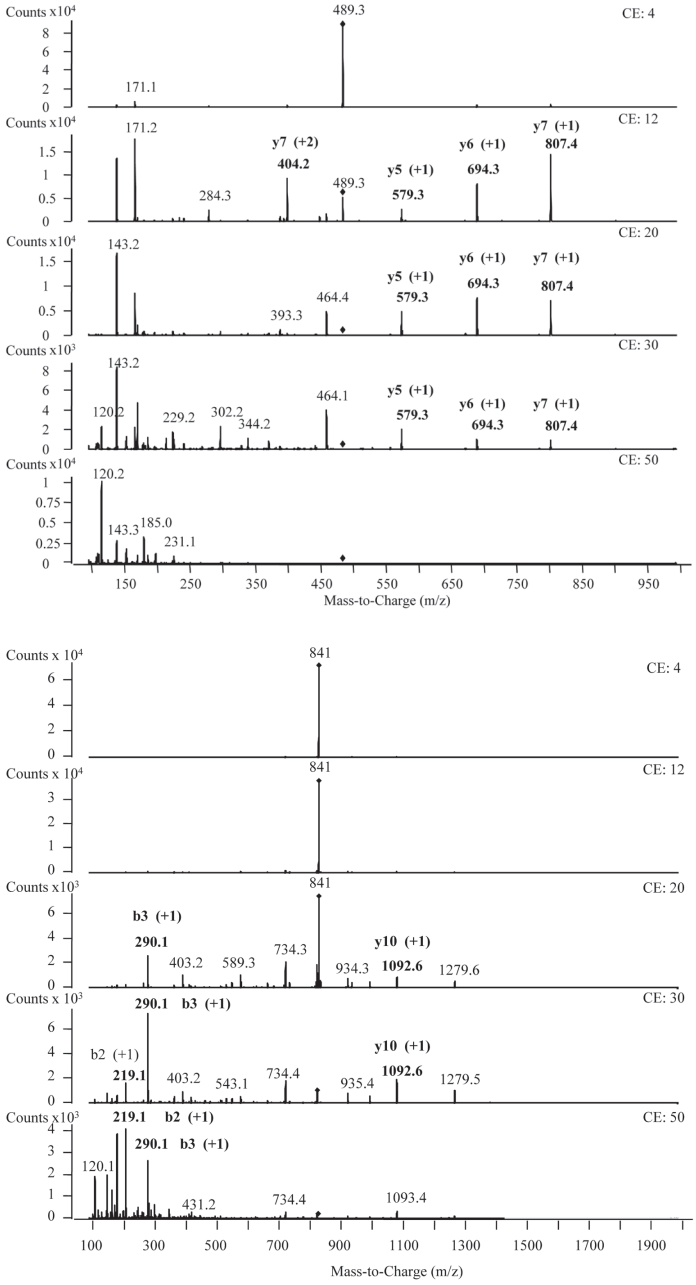

Figure 3. Top panel: Product ion scan of peptide AVIDDAFAR (parent ion: 489.3(+2); ♦) at different collision energies (CE) of 4, 12, 20, 30, 50 volt (from top to bottom). Lower panel: Product ion scan of peptide AFALWSAVTPLTFTR (parent ion: 841 (+2); ♦) at different collision energies (CE) of 4, 12, 20, 30, 50 volt (from top to bottom). MS/MS conditions were further optimized with the Agilent MassHunter Optimizer Automated MS Method Development Software to arrive at the final MMP-9 SRM assay (see **Table 2** for details).

3.2. Development of a quantitative SRM assay for MMP-9 in BALF

In order to find the best compromise between selectivity and sensitivity, we evaluated the effect of changing the width of the selection window for parent (Q1) and fragment ions (Q3) for AVIDDAFAR between ±0.35 u, ±0.6 u, and ±1.25 u while monitoring the peak area of the quantifier ion (y7 (807.4)) in the absence and presence of BALF. To this end, a pool of BALF samples from lung transplantation patients was spiked with 3.6 ng/mL rec. MMP-9 CD, chloroform-methanol precipitated and trypsin digested. To determine the effect of changing the resolution of Q3, we maintained Q1 at unit resolution. No interference was detected even when Q3 was opened to ±1.25 u while sensitivity increased by a factor of 2.4. Q3 was thus set to this resolution while varying the transmission window of Q1 for parent ion selection. No interferences from the biological matrix (BALF) were observed when using a width of ±0.6 u while sensitivity increased by another factor of 2.5. When widening parent ion transmission to ±1.25 u the response of the stable isotope-labeled internal standard peptide differed significantly from the non-labeled peptide indicating interference of components from the biological matrix with the standard. For further development of the assay, we therefore used a width of ±0.6 u for Q1 and ±1.25 for Q3.

Based on previous observations that peptides tend to adsorb to autosampler vials, [32] tryptic digests of the catalytic domain of rec. MMP-9 CD were diluted in 5% FA/ 5% DMSO to establish a calibration curve in the absence of BALF and to assess the potential of these peptides for quantitative work. By monitoring the transitions as given in **Table 2,** AVIDDAFAR gave a linear response without correction based on the stable isotope-labeled internal standard peptide ($y = 480.55 x + 718.87$, $r^2 = 0.9962$; **Figure 4A**).

Transitions for AFALWSAVTPLTFTR were also detected at all concentration levels but with poorer linearity ($r^2 = 0.8164$) indicating problems with peptide adsorption (**Figure 4C**). Correlation between peptide concentration and signal intensity for this peptide improved when adding trypsin-digested rec. MMP-9 CD to BALF, mostly likely since the excess of peptides derived from digested BALF proteins competed with the signature peptide for the available adsorption sites ($r^2 = 0.9112$) (**Figure 4D**).

Due to the better response of AVIDDAFAR as compared to AFALWSAVTPLTFTR in BALF (**Figures 4B and 4D**), we based our quantitative results on AVIDDAFAR, while detection of AFALWSAVTPLTFTR was used as confirmatory evidence for the presence of endogenous MMP-9.

Quantification of MMP-9 in BALF requires sample preparation notably to remove the highly abundant phospholipids [33], which interfere with electrospray ionization [34, 35]. To assess repeatability of sample preparation using chloroform-methanol precipitation of proteins, three separate aliquots of BALF from lung transplant patients were spiked with rec. MMP-9 CD at 1.4 ng/mL (appr. 2-times LLOQ; see Materials and Methods for details) and measured by LC-MS/MS in the SRM mode. The peak area of the quantifier ion of AVIDDAFAR (y7: 807.4) showed a standard error of +/- 8.2%, indicating that sample preparation is highly repeatable. Protein recovery after chloroform-methanol precipitation was excellent (starting protein concentration: 36.2 µg/mL; protein concentration after sample preparation: 36.1 +/- 0.5 µg/mL).

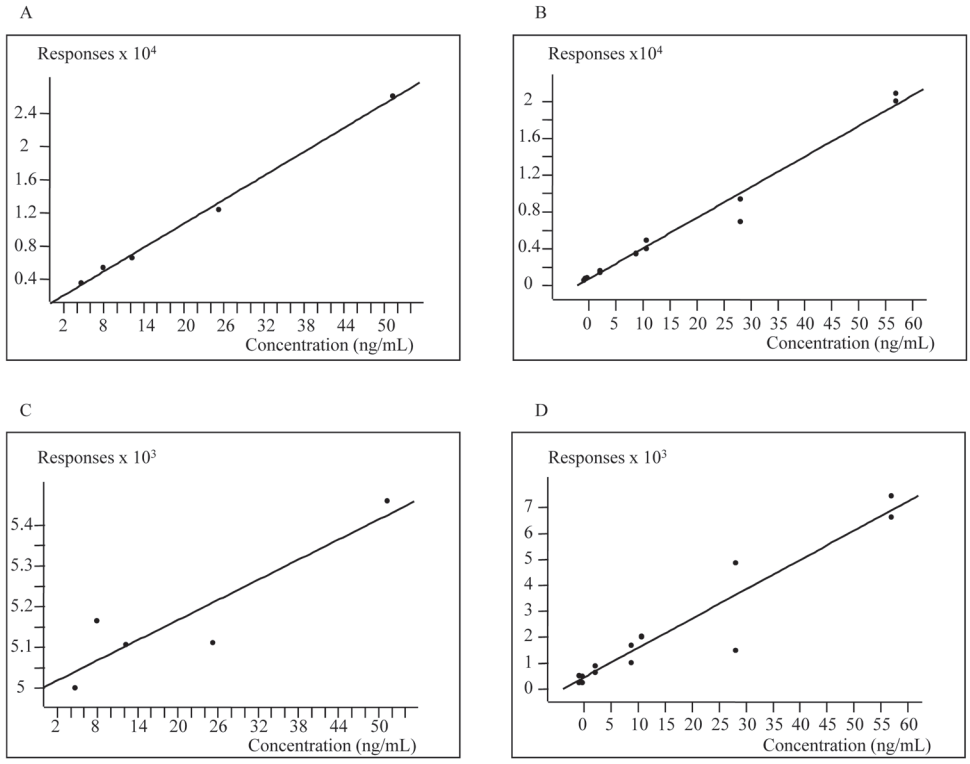

Figure 4.

A: Calibration curve of peptide AVIDDAFAR (parent ion: 489.3(+2)) from rec. MMP-9 CD injected in 5%FA/5%DMSO between 5.2ng/mL and 52ng/mL. Quantifier ion: 807.4 (y7 (+1)). Qualifier ions: 694.3 (y6 (+1)), 579.3 (y5 (+1)) and 404.2 (y7 (+2)); y = 480.55 x + 718.87, r^2 = 0.9962

B: Calibration curve of peptide AVIDDAFAR (parent ion: 489.3(+2)) from trypsin-digested rec. MMP-9 CD in BALF at 8 levels between 0 – 57.8 ng/mL. Quantifier ion: 807.4 (y7 (+1)). Qualifier ions: 694.3 (y6 (+1)), 579.3 (y5 (+1)) and 404.2 (y7 (+2)). y = 331.63 x + 418.60, r^2 = 0.9801

C: Calibration curve of peptide AFALWSAVTPLTFTR (parent ion: 841 (+2)) from rec. MMP-9 CD injected in 5%FA/5%DMSO between 5.2 ng/mL and 52 ng/mL. Quantifier ion: 1092.3 (y10 (+1)), Qualifier ions: 290.1 (b3 (+1)), 219.2 (b2 (+1)); y = 8.25 x + 4995.38, r^2 = 0.8164

D: Calibration curve of peptide AFALWSAVTPLTFTR (parent ion: 841 (+2)) from trypsin-digested rec. MMP-9 CD in BALF at 8 levels between 0 – 57.8 ng/mL. Quantifier ion: 1092.3 (y10 (+1)), Qualifier ions: 290.1 (b3 (+1)), 219.1 (b2 (+1)); y = 113.11 x + 347.09, r^2 = 0.9112

Since the pooled BALF sample contained a low but detectable amount of MMP-9 of 0.6 ng/mL, this amount was subtracted from all measured rec. MMP-9 CD levels to calculate the accuracy. Due to endogenous MMP-9 in the pool of BALF used for the calibration curve, the lowest points (0.15 and 0.5 ng/mL) show poor accuracy. The lower limit of accurate quantification was thus set at 2.9 ng/mL (SNR ~ 35). **Table 3** shows that the bias of the method ranged between 0.4 and 21% above the LLOQ of 2.9 ng/mL. To determine the lower limit of detection (LLOD) the SNR was set to 3. This resulted

in an LLOD of ~250 pg/mL (about 3 pM for a protein of ~90 kDa). Considering that 17.7 µL were injected per calibration point, this corresponded to on-column amounts of 50 pg MMP-9 (~560 amol) at the LLOQ and 4.4 pg MMP-9 (49 amol) at the LLOD. Injection of 5% FA/5% DMSO between analyses showed that there was no detectable carry-over.

Spiked MMP-9 in BALF (ng/mL)	Measured MMP-9 in BALF (ng/mL)	Mean MMP-9 in BALF (ng/mL)[1]	Calculated bias (%)[2]
0	0.6	0.6 (0)	not applicable
	0.5		
0.15	1.1	1.0 (0.4)	180
	0.9		
0.5	1.4	1.4 (0.8)	56
	1.3		
2.9	3.6	3.3 (2.7)	7.3
	3.0		
9.6	9.4	9.3 (8.7)	9.2
	9.2		
11.6	13.7	12.2 (11.6)	0.4
	10.8		
28.9	27.1	23.4 (22.8)	21.2
	19.6		
57.8	59.2	60.5 (59.8)	3.6
	61.7		

Table 3. Determination of the accuracy of measuring MMP-9 in BALF by LC-MS/MS in the SRM mode based on peptide AVIDDAFAR (parent ion: 489.3 (+2)). Quantification was based on the quantifier ion (807.4 (y7 (+1)) and three qualifier ions were monitored: 694.3 (y6 (+1)), 579.3 (y5 (+1)), 404.2 (y7 (+2)). Based on these analyses an LLOQ (35-times the SNR) of 2.9 ng/mL and an LLOD (3-times the SNR) of 0.25 ng/mL were determined. Note that there was a low level of endogenous MMP-9 (0.6 ng/mL) in the pooled BALF.

3.3. Analysis of BALF from lung transplantation patients

10 BALF samples (10 µg total protein) from patients having undergone lung transplantation were analyzed and quantified. The BALF samples showed considerable variation in total protein concentration (**Table 1**). Each sample was prepared in duplicate and an equivalent of 4 µg trypsin-digested total protein from each preparation was subjected to microfluidics-based nanoLC-MS/MS in the SRM mode.

1 Values in brackets are corrected by subtracting the level of endogenous MMP-9 (0.6 ng/mL) from the measured values.

2 Accuracy was calculated based on the corrected concentrations.

MMP-9 was quantifiable in 4 out of the 10 BALF samples above the LLOQ based on the expected transitions, their ratios and co-elution with the internal standard peptide (**Table 4** and **Figure 5**). One sample (404) showed an endogenous MMP-9 level slightly below the LLOQ. MMP-9 was detectable in 3 other BALF samples above the LLOD with an SNR between 3 and 10 and co-elution with the internal standard peptide but the ratio between parent ion and fragment ions did not meet the criterion of being within +/- 25% of the expected values (**Table 4** and **Figure 5**). MMP-9 detection was confirmed by monitoring the transitions for AFALWSAVTPLTFTR and its stable isotope-labeled internal standard peptide.

Sample	Acute rejection	Disease	Total protein concentration µg/ML	Measured MMP-9 (ng/mL)	Average of MMP-9 measured (ng/mL)	Average of MMP-9 measured normalized to 1 mg of total protein	IL-8 (pg/mL)	IL-6 (pg/mL)
396 R1	No	COPD	124.1	16.32	15.64	126.13	121	12.3
396 R2				14.97				
395 R1	No	COPD	123.9	8.93	8.65	69.75	401	10.2
395 R2				8.38				
389 R1	Yes	COPD	88.5	6.77	6.76	76.38	720	3
389 R2				6.75				
390 R1	No	COPD	185.1	7.62	7.24	39.11	1127	48.5
390 R2				6.86				
404 R1	Yes	CF	92.3	2.21	2.21	24.05	79.4	2
404 R2				2.22				
394 R1	No	COPD	93.5	-[1]	-[1]	-[1]	42.3	2.8
394 R2				-[1]	-[1]	-[1]		
406 R1	Yes	IPF	49	-[1]	-[1]	-[1]	7.3	2
406 R2				-[1]	-[1]	-[1]		
386 R1	Yes	COPD	28.1	-[1]	-[1]	-[1]	5.4	2
386 R2				-[1]	-[1]	-[1]		

Table 4. Analysis of MMP-9 in BALF from lung transplantation patients based on peptide AVIDDAFAR (parent ion: 489.3 (+2)). Quantification was based on the quantifier ion 807.4 (y7 (+1) and three qualifier ions were monitored: 694.3 (y6 (+1)), 579.3 (y5 (+1)), 404.2 (y7 (+2)). Four of the 10 analyzed BALF samples showed endogenous MMP-9 above the LLOQ (2.9 ng/mL) and met all the criteria for quantification by SRM. One sample (404) showed an endogenous MMP-9 level slightly below the LLOQ. The level of MMP-9 in 3 of the samples was detectable above the LLOD but with ratios between qualifier and quantifier fragment ions that were outside of the allowed +/- 25% of the expected values. However, the detected target peptide co-eluted with the internal standard peptide, so we include them as tentative results in this table (indicated by [1]). Measurements of Interleukin-8 and -6 (IL-8, IL-6) in BALF was performed by a chemiluminescent immunoassay (see section 2.4).
R1: Repetition 1; R2: Repetition 2.

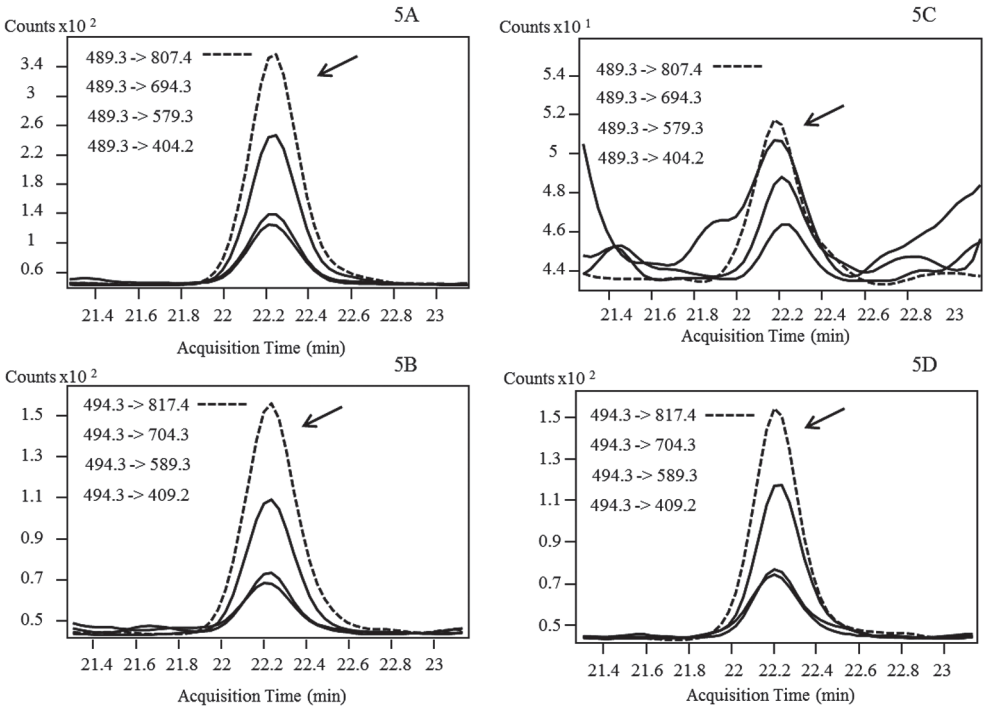

Figure 5. LC-MS/MS analysis (SRM mode) of MMP-9 above the LLOQ in BALF from a lung transplant patient with no complications due to acute rejection (sample 396; see **Table 1**). **(A)** Extracted ion chromatograms of the transitions for the target peptide AVIDDAFAR (489.3→404.2/579.3/694.3/807.4) and **(B)** for the corresponding transitions of the stable-isotope-labeled internal standard peptide (494.3→409.2/589.3/704.3/817.4) show co-elution at the expected retention time (see arrows) (the transitions 489.3 to 807.4 and 494.3 to 817.4, represented as dashed lines, were used for quantification).
LC-MS/MS analysis (SRM mode) of MMP-9 between the LLOQ and the LLOD in BALF from a lung transplant patient with no complications due to acute rejection (sample 394; see **Table 1**). **(C)** Extracted ion chromatograms of the transitions for the target peptide AVIDDAFAR (489.3→404.2/579.3/694.3/807.4) (4C) and **(D)** for the corresponding transitions of the internal standard peptide (494.3→409.2/589.3/704.3/817.4) show co-elution at the expected retention time (see arrows) (the transitions 489.3 to 807.4 and 494.3 to 817.4, represented as dashed lines, were used for quantification).

The level of MMP-9 in BALF did not correlate with the levels of IL-8, a chemotactic cytokine for neutrophils that can also activate NFκB, or IL-6, a cytokine related to the acute-phase response of the body (**Table 3**) nor with acute rejection of the transplanted lung. Concentrations of these cytokines were previously shown to be non-predictive [28, 36].

3.4. ELF analysis.

We investigated the applicability of the developed SRM assay to a related biological matrix, epithelial lining fluid (ELF) collected by bronchoscopic microprobes. Analysis of a calibration curve with 5 levels of spiked MMP-9 CD (1.9 ng/mL, 5.3 ng/mL, 10.7 ng/mL, 16 ng/mL and 33.3 ng/mL) showed that sample preparation and LC-MS/MS analyses are also applicable to ELF (r^2 =0.95 for AVIDDAFAR). Four ELF samples were analyzed: 2 samples from COPD (Chronic Obstructive Pulmonary Disease) patients, 1 control sample and 1 sample from a pool of 2 lung transplantation patients. ELF from the lung transplantation patient showed a strong response of the monitored transitions of AVIDDAFAR and of AFALWSAVTPLTFTR. The monitored transitions had the correct ratios of intensity relative to the qualifier ion and the correct retention time. One COPD sample had a signal slightly above the LLOD. The other 2 samples did not contain detectable amounts of MMP-9.

4. Discussion and conclusion

Current developments in the area of biomarker research emphasize that proteome-wide discovery studies must be followed up by targeted quantitative validation that allow analyzing a larger number of samples. While immunochemical assays (ELISA) are currently the most widely used approach, there is an increasing tendency to complement or even replace them by analytical techniques that provide direct chemical information about the analytes such as LC-MS/MS. A major challenge for LC-MS/MS is its lower concentration sensitivity when compared to ELISA, while it has clear advantages when it comes to providing more reliable qualitative and quantitative information.

To address these issues, liquid chromatography in combination with tandem mass spectrometry in the selected reaction monitoring (SRM) mode can provide high selectivity and sensitivity in complex biological samples. SRM assays have, to date, been mainly applied to serum and plasma, where they are generally limited to the low µg/mL range unless immunoaffinity enrichment is used. Most potential serum biomarkers are, however, at the low ng/mL or even pg/mL level, which means that sample preparation forms a critical part of any targeted LC-MS/MS method in the SRM mode. Keshishian *et al.* demonstrated that multiplexed SRM assays allow detecting proteins at the 1-20 ng/mL range after removal of the 12 most abundant proteins by immunoaffinity depletion and fractionation by strong cation-exchange chromatography (SCX) [20, 21]. However, most high-sensitivity SRM assays in serum or plasma require antibody-based protein enrichment, which suffers from similar drawbacks as ELISA namely that highly specific antibodies must be available or generated and validated, which renders assay development costly and often lengthy. MMP-9 has been analyzed in mouse serum by immunoaffinity LC-MS/MS in the SRM mode reaching a sensitivity of 30 pM (2.7 ng/mL) [16].

While SRM assays for proteins in serum or plasma are becoming more widely used, there is, to our knowledge, currently no description of such assays for proteins in BALF. BALF is however more meaningful than serum for the discovery and/or the validation of potential biomarkers related to

pulmonary diseases. There is thus a need to study this matrix by targeted analysis. Although BALF shares certain characteristics with other biofluids, it differs significantly in that it contains a high level of phospholipids, which interfere strongly with LC-MS/MS in the electrospray ionization mode. At the same time protein concentration in BALF is about 1000-fold lower than in serum or plasma meaning that larger volumes must be treated in order to introduce comparable protein amounts into the analytical system.

Next to targeted analyses by SRM there have been a number of proteomics studies in human BALF of which the one by Wu *et al.* remains the most extensive one [37]. Due to the wide dynamic range of the BALF proteome, a range of prefractionaton and sample preparation steps were required to detect MMP-9 at the low ng/mL level including concentrating BALF by ultrafiltration after depletion of the six most abundant proteins by immunoaffinity chromatography. The final steps comprised gel electrophoresis in the presence of SDS and in-gel tryptic digestion making this a very lengthy and work-intensive procedure. Another shortcoming of such a complex experimental approach is the possibility of protein loss during sample preparation. The time required for sample preparation and the number of fractions that are generated per sample are not compatible with high-throughput analysis.

The SRM assay described in this study is designed for quantification of low abundant proteins in BALF samples without extensive fractionation. Our SRM assay can quantify endogenous MMP-9 with an LLOQ of 2.9 ng/mL at an SNR of ~35. MMP-9 in BALF of lung transplantation patients with and without complications due to acute rejection of the transplant was measured but no correlation was found. Other indicators of an inflammatory response such as IL-8 and IL-6 or the infiltration of neutrophils or lymphocytes did also not allow predicting the occurrence of acute rejection. We are currently extending our assay to combine it with the enrichment of active MMP-9 on an immobilized inhibitor material [38-41] to investigate whether active MMP-9 or MMP-9 – TIMP-1 complexes may be predictors of acute rejection after lung transplantation.

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Chapter 4

Studying the MMP-9 – TIMP-1 protease-inhibitor system by activity-based enrichment and selected reaction monitoring (SRM) nano-LC-MS/MS

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Abstract

Deregulation of Matrix Metalloprotease (MMP) activity may lead to numerous pathological events (e.g. metastasis, chronic inflammation). Analysis of MMP activity in relation to the level of endogenous inhibitors is thus important for correlating the balance between MMP activation and inhibition to biological mechanisms and pathophysiology. A number of studies have demonstrated the use of targeted LC-MS/MS in the SRM (Selected Reaction Monitoring) mode for the multiplexed analysis of proteins. However, SRM assays have so far never been used to study protease-inhibitor systems and their balance.

In the current work, we designed SRM assays to study MMP-9 and TIMP-1, its major endogenous inhibitor, by selecting signature peptides from the pro-domain and the catalytic domain of the enzyme as well as from TIMP-1. The multiplex LC-MS/MS method was applied to study the protease-inhibitor balance in supernatant of human epithelial cells that were exposed to cigarette smoke extract (CSE). Our results showed that there is a considerable molar excess of TIMP-1 over MMP-9 in cell culture supernatant and that no free, active MMP-9 was detectable based on the molar ratio between signature peptides from the pro-domain and the catalytic domain even after CSE stimulation. However, the molar ratio between TIMP-1 and proMMP-9 decreased significantly when human epithelial cells were exposed to CSE due a lower concentration of TIMP-1.

To determine whether there is a minor amount of free, active MMP-9 in the cell culture supernatant or whether all active enzyme is inhibitor-bound, we combined the SRM assays with an activity-dependent affinity enrichment step using immobilized broad-spectrum MMP inhibitors. While active MMP-9 was easily detectable after chemical activation with APMA (4-aminophenylmercuric acetate) after blocking the excess of TIMP-1 by pre-incubation with active MMP-12, it was not possible to detect endogenous active MMP-9 after affinity enrichment. This indicates that there is indeed no free active MMP-9 in the supernatant when exposing epithelial cells to CSE.

Our work opens the possibility to assess the stoichiometry between pro-MMP-9, active MMP-9 and TIMP-1 in a complex biological system by targeted LC-MS/MS in the SRM mode, an approach that may be combined with activity-dependent enrichment techniques. The described analytical strategy may be extended to other protease-inhibitor systems to allow a more comprehensive analysis of protease activation and inhibition under different biological conditions.

1. Introduction

Human Matrix-Metalloproteases (MMPs), a family of 23 enzymes, are highly regulated *in vivo* [1-3]. MMPs are secreted in their inactive, pro-form (also called zymogen form) and must undergo a number of controlled binding and proteolysis events to become catalytically active, following removal of the pro-domain. Nature developed a number of tight-binding inhibitors, the Tissue Inhibitors of MetalloProteases (TIMPs) that fulfill this task [1, 4]. The blood-based broad-spectrum inhibitor α_2-macroglobulin may be considered to serve as a 'back up system' to prevent the uncontrolled release of active proteases into the systemic circulation. Any disturbances of this tight regulation may lead to pathologies related to the degradation of extracellular matrix proteins (such as collagen, proteoglycans and fibronectin) with subsequent tissue remodeling as well as the release of cytokines, growth factors and chemokines [1-4]. It is thus critical to understand the regulation of MMPs in relation to the regulation of TIMPs (the so-called 'protease-anti-protease balance') in order to gain a better understanding of the role of MMPs in biological systems and notably in relation to disease development.

Numerous approaches have been developed to measure protease activity. One of the most sensitive assays is zymography which is based on the use of natural MMP substrates that are embedded in a polyacrylamide gel allowing detection of the gelatinases MMP-2 and MMP-9 in the low pg range [5, 6]. Another assay, developed by Verheijen *et al.* [7], is based on the use of an engineered substrate, pro-urokinase modified with a short peptide linker RPLGIIGG, which is a target for active MMPs. Cleavage of the engineered pro-urokinase by MMPs within biological samples is directly proportional to the activation of urokinase. Hanemaaijer *et al.* adapted this method into an MMP-9-specific activity assay (with a sensitivity similar to gelatin zymography) by combining it with an immunocapture step [8]. Although both techniques have been used to detect endogenous active enzyme, they suffer from a number of shortcomings.

Zymography is almost exclusively used with gelatin as co-polymerized substrate to detect MMP-2 and MMP-9. Furthermore, pro-MMPs and TIMP/MMP complexes are also detected due to the denaturation-renaturation steps during the zymography procedure. While pro-MMPs may be differentiated from the active forms based on a different molecular weight, the often quite abundant TIMP/MMP complexes dissociate and cannot be distinguished from free, active MMPs. The activity-based immunocapture assay relies on recognition of a given MMP by an antibody, cleavage of the engineered pro-urokinase and the subsequent measurement of urokinase activity using a synthetic pro-fluorescent substrate. Antibody binding must thus be specific and not interfere with proteolytic activity. These rather stringent criteria make it hard to develop assays for the entire enzyme family, which limits the multiplexing capacity.

The use of activity-based probes (ABPs) as pioneered by Cravatt *et al.* for serine hydrolases [9, 10] and later extended to metalloproteases [11-14] relies on the interaction between chemically synthesized MMP inhibitors with the active site of the enzymes. In case the inhibitors contain a tag, active enzymes may be enriched, for example through the biotin-avidin interaction. Enrichment may be

followed by subsequent chemical analysis, in some cases based on mass spectrometry. Alternatively synthetic inhibitors may be immobilized on stationary phases and used to enrich active enzymes by activity-dependent affinity chromatography [15-20].

The ABP approach is based on the use of a synthetic probe carrying three fundamental moieties: an element to recognize the active site of the enzyme, a reactive group to label the targeted protein covalently and a reporter-tag to enrich and detect the labeled enzyme [12, 21, 22]. This design has been successfully applied to enzyme families such as cysteine proteases, serine hydrolases, and proteasome subunits [12, 22]. For these studies, labeling is based on the use of an electrophilic group in the probe that reacts with a nucleophilic functional group which is part of the enzyme's active site (e.g. the hydroxyl group of serine or the thiol group of cysteine) to form a covalent bond with the active target enzymes. The catalytic properties of MMPs, which are based on the activation of a water molecule in the active site through interaction with a Zn^{2+} ion, prevent such an approach [12, 21]. A photoreactive group (aryl azide, diazirine or benzophenone) carried by the probe is needed to introduce a covalent bond with the targeted protein upon irradiation. This ABP technique has been investigated in several studies for a panel of MMPs and demonstrated labeling of the active enzyme but not of the pro-form or TIMP-enzyme complexes [11, 13, 23]. However, labeling proves to be incomplete and the efficiency of labeling varies considerably between different MMPs. Dive *et al.* reported a labeling efficiency of ~1% for MMP-8 while MMP-12 was labeled to ~40% [23, 24]. Such efficiency differences make comparative, quantitative studies concerning the activation and inhibition of MMPs in complex biological samples difficult even if compensated for by the inclusion of stable-isotope-labeled internal standards. To tackle this problem, Cravatt *et al.* synthetized a library of ~20 synthetic probes (a so-called cocktail of probes) where the tag was added to the covalently labeled MMPs in a second step using click chemistry to avoid a negative effect of the tag on the inhibition and labeling of active enzymes [14]. While spiked MMPs were detected down to 100 ng/mg total protein in a lysate of mouse liver (10 ng/mg for MMP-1), no endogenous, active MMPs were detected in the studied human cell lines MUM-2B, MUM-2C, MDA-MB-231, MCF7. However, detection of other active members of the metalloprotease family such as ADAM-10, ADAM-17, AlaAp, Neprilysin and AFG3 were shown [14]. It should be noted that all of these enzymes are membrane proteins and that no soluble, active matrix metalloprotease was detected.

The work of Bregant *et al.* compared the ABP technique to the enrichment of spiked, active MMPs that had interacted with a biotinylated inhibitor via streptavidin affinity chromatography [25]. Two different probes, one with and one without a photoactivatable group, were tested to compare enrichment of active MMPs. Spiked samples were first incubated with the biotinylated, synthetic inhibitors until full inhibition of the added active MMPs was reached (no more protease activity based on the conversion of profluorescent substrates) before proceeding to the enrichment of the inhibitor-MMP complexes. The amount of the probes was adjusted according to their respective IC_{50} values. Affinity enrichment of the spiked enzymes without photocrosslinking was almost complete while photoaffinity capture was only partial, since not all of the added active enzymes were labeled.

This comparative study highlights the benefit of the affinity enrichment approach over the ABP strategy.

Freije *et al.* [16] discussed activity-based enrichment in comparison to the ABP strategy. The work of Freije *et al.* demonstrated further the benefit of using immobilized inhibitors in fully automated equipment for the enrichment of active MMPs [26]. Such an approach is particularly interesting for the analysis of body fluids in clinical studies. Additional work from our group and others gave further credit to this approach. The work of Hesek *et al.*, using a similar strategy, showed multiplexing capacity by enriching active MMP-2 and MMP-14 from carcinoma tissue extracts as revealed by zymography and western blot analysis [27] and the work of Geurink *et al.* showed enrichment of ADAM-17 from A549 cells in culture [20].

While a wide range of methods has been developed and tested for the activity-dependent analysis of metalloproteases and notably MMPs, there is a need to develop non-biased, quantitative and selective analytical methods to assess the activity status of MMPs in complex biological matrices. An ideal assay should allow the absolute quantification of proteases and inhibitors in a highly specific and sensitive manner for any MMP or TIMP preferably in a multiplex assay format.

In the past decade absolute protein quantification by mass spectrometry in the SRM (Selected Reaction Monitoring) mode has gained considerable interest for the validation of potential biomarkers and the quantification of biopharmaceutical proteins [28, 29]. The relatively low cost for developing a new SRM assay, its high specificity due to the choice of specific signature peptides and the use of identical, synthetic, stable isotope labeled-peptides as internal standards (IS) as well as the multiplexing capacity of modern mass spectrometers have all contributed to this growing success [28-34].

LC-MS/MS in the SRM mode has so far never been used to study interconnected enzyme-inhibitor systems although it should be ideally suited to this task based on its multiplexing capacity and selectivity. In the current work, we demonstrate the feasibility of LC-MS/MS in the SRM mode to study protease-inhibitor systems by quantifying MMP-9 (pro-form and catalytic domain) and TIMP-1, its major inhibitor, in the supernatant of human epithelial cells that were exposed to cigarette smoke extract (CSE). To determine whether the processed form is free or inhibitor-bound we combined this strategy with an approach where samples are first subjected to activity-based enrichment on an immobilized, synthetic inhibitor followed by SRM analysis.

2. Material and methods

2.1. Material

Ammonium bicarbonate (AB), dimethyl sulfoxide (DMSO), chloroform, dithiothreitol (DTT), formic acid (FA), ethanolamine, amino phenyl mercuric acetate (APMA), gelatin from porcine skin type A, calcium chloride, ammonium persulfate (APS), sodium acetate, and iodoacetamide (IAA) were purchased from Sigma Aldrich. Trypsin (sequencing grade, cat. n° V5111) was from Promega. NaCl, glacial acetic acid, sodium thiosulfate, and sodium carbonate were obtained from Merck. Methanol

and acetonitrile (ACN) were purchased from Biosolve and formaldehyde from JT Baker. Sodium dodecyl sulfate (SDS), tris(hydroxymethyl)aminomethane (Tris), glycine, ethylenediaminetetraacetic acid (EDTA), and silver nitrate were purchased from Duchefa, Brij-35 was from Janssen Chemica, and glycerol from Genfarma BV. A 30% acrylamide/bis-acrylamide (29:1) solution, Coomassie brilliant blue G-250, bromophenol blue, and tetramethylethylenediamine (TEMED) were from Biorad. Amicon Ultra-0.5 centrifugal devices (10 kDa cut-off) were purchased from Millipore, Benzonase from Novagen, the pro-fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 from Bachem, and NHS-activated Sepharose 4 Fast Flow from GE Healthcare. Reagents related to the cell culture experiments were as follows: minimal essential medium, penicillin, streptomycin, L-glutamine, gentamicine, fungizone (GIBCO), cell culture plates (Greiner Bio One), F12 (Ham's F12) (Lonza BioWhittaker), collagen (Purified Bovine Collagen Solution Advanced) (Biomatrix) and fibronectin and bovine serum albumin (Sigma).Two synthetic inhibitors with a free amine for immobilization were used for this study: TAPI-2 from Calbiochem and ML-5 that was synthesized according to Leeuwenburgh *et al.* [19]. Ultrapure water (resistivity of 18.2 MΩ·cm) was obtained from a Sartorius Stedim water purification system (model 611 VF). Protein markers for SDS-PAGE and zymography analyses were from Invitrogen (10747-012). The following stable isotope-labeled internal standard peptides were obtained from JPT Peptide Technologies (http://www.jpt.com): AVIDDAFARXSAZ, YVDINTFRXSAZ, AFALWSAVTPLTFTXSAZ (X= $R^{13}C_6$, 97-99%; $^{15}N_4$, 97-99%, Z=JPT-Tag, which is removed during tryptic digestion) and AVIDDAFAR*(* K or R: $^{13}C_6{}^{15}N_2$), SEEFLIAGK*, GFQALGDADDIR*, QLAEEYLYR*, QLSLPETGELDSATLK* were purchased from New England Peptide. Pro-MMP-9 and TIMP-1 were obtained from R&D Systems. MMP-9 CD (Catalytic Domain) without the fibronectin type II-like repeats (residues 107-216 fused to residues 391-443 with an additional M-G dipeptide at the N-terminus) expressed and purified according to the protocol of Shipley *et al.* and MMP-12 CD G_{106} to N_{268} and ^{15}N-MMP-12 CD G_{106} to N_{268} expressed and purified according to the protocol of Parkar *et al.* were a gift from AstraZeneca [35, 36].

2.2. Culture supernatant from 16HBEo- cells

Human bronchial epithelial cells (16HBEo-) were cultured in Minimal Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS), 5 mg/mL streptomycin, 5000 units/mL penicillin, 200 mM L-glutamine, 10 mg/mL gentamicine and 250 µg/mL fungizone. Cell culture plates were coated with F12 cell culture medium supplemented with 1 mg/mL bovine serum albumin, 2.9 mg/mL collagen and 1 mg/mL fibronectin. Cells were grown up to confluence before proceeding to the stimulation with 10% cigarette smoke extract (CSE).

Prior to stimulation, cells were incubated overnight in FBS-free MEM containing 5 mg/mL streptomycin, 5000 units/mL penicillin, 200 mM L-glutamine, 10 mg/mL gentamicine and 250 µg/mL fungizone. Cells were stimulated with 10% CSE for 24 h or with medium without CSE (CTR) after which cell culture medium was collected. 16HBEo-cell culture supernatant was centrifuged at 2000 g for 5 min (4 ºC) before being aliquoted and stored at -80 ºC until use.

CSE was prepared by using a peristaltic pump (Watson Marlow 323 E/D). The smoke of two cigarettes (University of Kentucky 2R4F) without a filter was bubbled through 25 ml of FBS-free MEM medium containing antibiotics and fungizone as described above. The obtained solution was diluted to 10% for the experiments.

2.3. BALF samples

A pool of BALF was constituted from 13 human BALF samples (8 lung transplantation patients and 5 patients with other pulmonary pathologies such as COPD or sarcoidosis). The procedures followed were in accordance with national and local ethical guidelines.

2.4. Determination of the total protein amount

Proteins in the cell culture supernatant were precipitated with chloroform-methanol according to the protocol of Wessel and Flugge [37] to remove phenol red, which interferes with the colorimetric μBCA assay. Briefly, samples (160 μL) were mixed with 600 μL methanol, 150 μL chloroform and 450 μL water by vortexing and centrifuged at 13600 g for 5 min. The pellet was washed with 450 μL methanol and centrifuged as described above. The dried protein pellet was dissolved in the original volume of PBS (Phosphate Buffer Saline; 140 mM NaCl, 9 mM Na_2HPO_4, 1.3 mM $Na_2H_2PO_4$, pH 7.4) supplemented with 2 % SDS by shaking for 10 min at room temperature. The total protein concentration was determined with the μBCA assay according to the manufacturer's instructions (Pierce). Absorbance was measured with a Fluostar Optima plate reader (BMG, Labtech) at 580 nm. BALF (50 μL) was diluted 3-fold with PBS to determine the total protein concentration with the μBCA assay as described for the cell culture supernatant.

2.5. Selection of peptides for SRM analysis

Two μg of TIMP-1 and 5 μg of pro-MMP-9 and MMP-12 CD were reduced with 4 mM DTT (20 min at 56 °C) and alkylated with 12 mM IAA (15 min at room temperature in the dark). Proteins were precipitated with chloroform-methanol as described above and the dried protein pellet was re-suspended in 20 μL of 50 mM ammonium bicarbonate. Proteins were digested with trypsin (enzyme to protein ratio 1:25 w/w) for 5 h at 37 °C. The peptide map of pro-MMP-9, MMP-12 CD and TIMP-1 (injection of ~600 fmol per run) was assessed in the MS1 mode as well as in the product ion scan mode on a triple-quadrupole mass spectrometer connected to a microfluidics-nanoLC system (see section on LC-MS/MS analysis). Selection of precursor and product ions was performed under the following conditions: fragmentor (FR) 135 V, dwell time 150 ms, collision energy (CE) 4, 12, 20, 30 or 50 V. Final optimization of the fragmentation conditions for the selected transitions was performed with steps of 10 V for FR and 2 V units for CE.

The selected peptides were subjected to sequence homology search against the UniProt Knowledgebase (*Homo sapiens*) consisting of UniProtKB/Swiss-Prot Release 2012_05 of 16-May-12 (20232 entries) and of UniProtKB/TrEMBL Release 2012_05 of 16-May-12 (110822 entries) using BLASTP to assure that they are unique for human MMP-9, human TIMP-1, and human MMP-12.

The selected peptides for pro-MMP-9 quantification contain a Glutamine (Q) residue at the N-terminus that might be subject to intramolecular rearrangement leading to pyro-glutamic acid (pyro-E). Modified peptides were obtained by resuspending the commercial IS in 100 mM ammonium bicarbonate for 24 h at room temperature and SRM conditions were optimized as for the non-modified peptides (injection of 1 pmol per run).

2.6. Sample preparation for the analysis of pro-MMP-9, total MMP-9, and TIMP-1 in cell culture supernatant

To assess the effect of CSE on 16HBEo-cells with respect to the secreted amount of MMP-9, pro-MMP-9 and TIMP-1, 10 µg of total protein in the cell culture supernatant (see **Figure 1** for a schematic overview of sample preparation) were submitted to buffer exchange (Amicon Ultra 0.5 mL centrifugal filter, 10 kDa as cut-off) into 50 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$ to digest DNA and RNA followed by incubation with 1 µL of Benzonase (Novagen) for 1.5 h at 4°C. One µL corresponds to an enzyme activity of 25 29 Units (one Unit is defined as the enzyme activity able to digest 37 µg DNA in 30 min in 50 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$). Proteins were reduced, alkylated and precipitated as described above. Proteins were trypsin-digested (enzyme to protein ratio 1:25 (w:w)) in the presence of stable isotope labeled internal standard peptides (IS) corresponding to 18 fmol for each of the targeted MMP-9 peptides and 126 fmol for each of the targeted TIMP-1 peptides (7.2 fmol and 50.4 fmol of these peptides were injected on the HPLC column, respectively). Tryptic digestion was stopped by adding 2.2 µL of 50 % FA / 50 % DMSO (Dimethylsulfoxide) and diluted twice (to a final volume of 44.4 µL) with 5 % FA / 5 % DMSO of which 17.7 µL were injected per sample and per calibrant point (4 µg of total digest per injection).

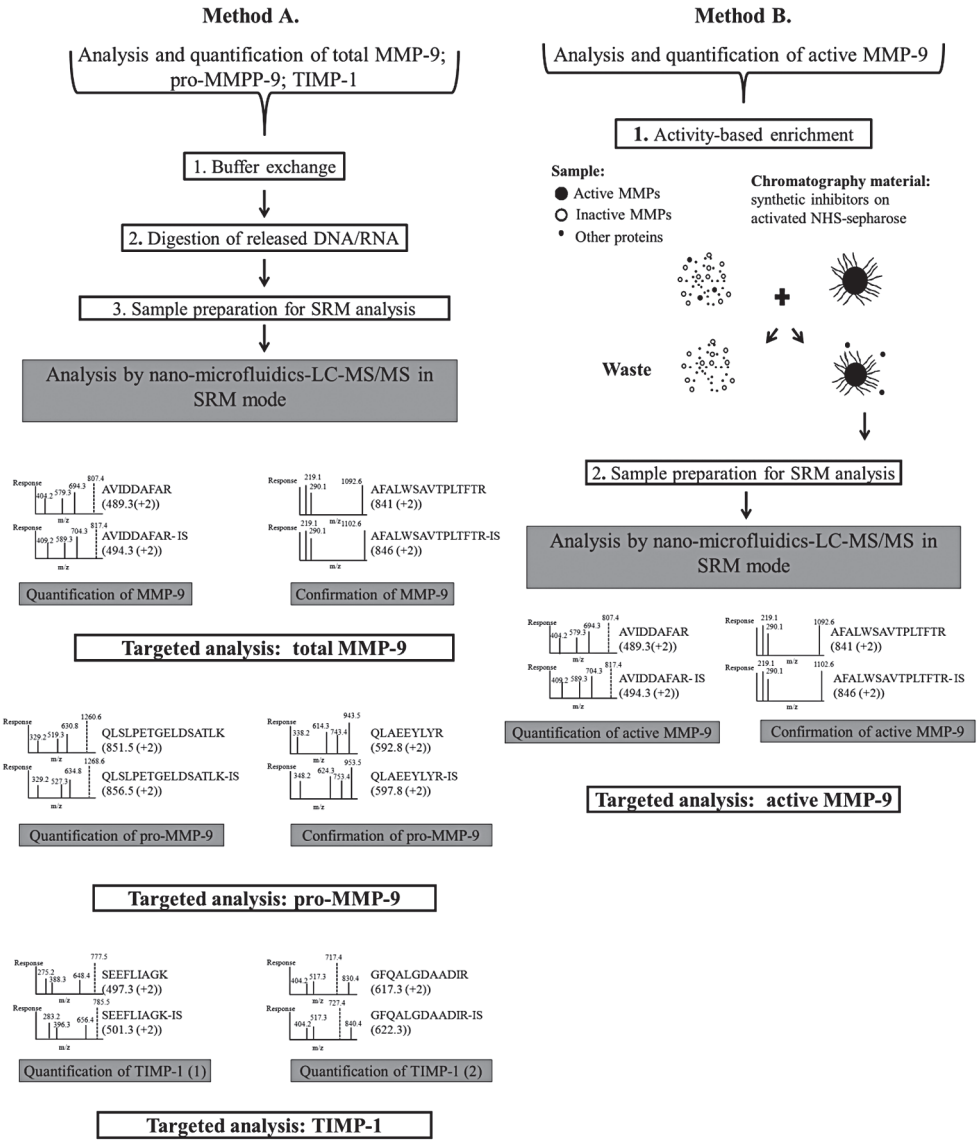
Method A.
Analysis and quantification of total MMP-9; pro-MMPP-9; TIMP-1
1. Buffer exchange
2. Digestion of released DNA/RNA
3. Sample preparation for SRM analysis
Analysis by nano-microfluidics-LC-MS/MS in SRM mode
AVIDDAFAR (489.3(+2))
AVIDDAFAR- IS (494.3 (+2))
AFALWSAVTPLTFTR (841 (+2))
AFALWSAVTPLTFTR-IS (846 (+2))
Quantification of MMP-9
Confirmation of MMP-9
Targeted analysis: total MMP-9
QLSLPETGELDSATLK (851.5 (+2))
QLSLPETGELDSATLK-IS (856.5 (+2))
QLAEEYLYR (592.8 (+2))
QLAEEYLYR-IS (597.8 (+2))
Quantification of pro-MMP-9
Confirmation of pro-MMP-9
Targeted analysis: pro-MMP-9
SEEFLIAGK (497.3 (+2))
SEEFLIAGK-IS (501.3 (+2))
GFQALGDAADIR (617.3 (+2))
GFQALGDAADIR-IS (622.3))
Quantification of TIMP-1 (1)
Quantification of TIMP-1 (2)
Targeted analysis: TIMP-1
Method B.
Analysis and quantification of active MMP-9
1. Activity-based enrichment
Sample:
Active MMPs
Inactive MMPs
Other proteins
Chromatography material: synthetic inhibitors on activated NHS-sepharose
Waste
2. Sample preparation for SRM analysis
Analysis by nano-microfluidics-LC-MS/MS in SRM mode
AVIDDAFAR (489.3(+2))
AVIDDAFAR- IS (494.3 (+2))
AFALWSAVTPLTFTR (841 (+2))
AFALWSAVTPLTFTR-IS (846 (+2))
Quantification of active MMP-9
Confirmation of active MMP-9
Targeted analysis: active MMP-9

Figure 1.

Method A: **Analysis and quantification of total MMP-9, pro-MMP-9, and TIMP-1 (left panel).**

Schematic overview of the sample preparation procedure for the quantification of total MMP-9, pro-MMP-9, and TIMP-1 in 16HBEo- cell culture supernatant by SRM analysis. Cell culture supernatant, which is mainly comprised of growth medium, was first exchanged to 'Benzonase buffer' (see Material and Methods) for the digestion of DNA and RNA by ultrafiltration at 10 kDa cut-off. Proteins were then reduced and alkylated, before being precipitated with chloroform-methanol. Precipitation allows desalting and concentrating the samples as well as denaturing proteins prior to trypsin digestion in the presence of stable-isotope-labeled internal standard peptides (these steps are called "Sample preparation for SRM analysis").

The following peptides were selected for SRM assays (total MMP-9: AVIDDAFAR (used for quantification), AFALWSAVTPLTFR (provided confirmatory evidence); pro-MMP-9: QLSLSPETGELDSATLK (used for quantification), QLAEEYLYR (provided confirmatory evidence); TIMP-1: SEEFLIAGK and GFQALGDAADIR (both peptides were used for quantification and to provide confirmatory evidence). The transitions represented with a dashed line are used for quantification.

Method B: Analysis and quantification of MMP-9 after activity-dependent enrichment (right panel).

The activity-based enrichment procedure is based on affinity chromatography using a synthetic inhibitor that is immobilized on NHS-activated Sepharose. Samples were first incubated with the immobilized inhibitor material to enrich active-MMP-9 before proceeding to the same steps (called "Sample preparation for SRM analysis") than in Method A. The SRM assay for active MMP-9 was based on AVIDDAFAR (for quantification) and on AFALWSAVTPLTFR (for confirmation).

Samples from two different passages for CSE and CTR cells were prepared in duplicate and quantified with a 6-point calibration curve prepared in duplicate by spiking 0, 10, 20, 30, 40, 60 ng/mL of TIMP-1 and 0, 0.5, 1.5, 2.5, 4.5, 6.5 ng/mL of pro-MMP-9 into a pool of cell culture supernatant that was constituted from equal aliquots of each experiment (see **Table 4**).

2.7. LC-MS/MS analysis

Samples were analyzed by nanoLC-MS/MS using a microfluidics (chip-cube) interface (Agilent, cat. n° G4240A) on a C-18 chip (Agilent, custom-made) with a 500 nL enrichment column (Zorbax 300 SB C-18, 5 µm) and a 75 µm × 150 mm separation column packed with the same chromatographic material. The interface contained a nanoelectrospray tip (2 mm length with conical shape: 100 µm OD × 6 µm ID) that was coupled on-line to a triple quadrupole mass spectrometer (Agilent, G6410B). Tryptic digests (4 µg total protein), dissolved in 5% formic acid (FA) / 5% DMSO, were loaded on the trap column in 0.1 % aq. FA, 3% acetonitrile at 2.5 µL/min via the autosampler (Agilent, cat. no. G1377A) equipped with an injection loop of 40 µL (Agilent, cat. no. G1377A) and a thermostated cooler (Agilent, cat. no. G1330B) maintaining the samples in the autosampler at 4° C. The interface was connected to an Agilent 1200 series HPLC system containing the following modules: nanopump (Agilent, cat. no. G2226A), capillary pump (Agilent, cat. no. G1376A) and solvent degasser (Agilent, cat. no. G1379B). The samples were enriched in the forward flushing mode (flushed with an extra 8 µL of 0.1% aq. FA, 3% acetonitrile using the capillary pump at 2.5 µL/min) after which the trapping column was switched in-line with the analytical column. Peptides were eluted with eluents A (0.1% FA in water) and B (0.1% FA in acetonitrile) at a flow rate of 0.25 µL/min using the following gradient program: 3% to 60% eluent B at 2%/min, 60% to 90% eluent B at 10%/min. Eluent B (90%) was

maintained for 10 min before returning to the starting conditions over 10 min. The column was equilibrated at the starting conditions for 8.5 min prior to the next injection. Carry-over was avoided by washing the needle for 10 s with 0.1% FA in 20 % methanol.

Samples were analyzed with unit resolution (± 0.35 u at half height) for the first and third quadrupole. Each transition was monitored with a dwell time of 20 ms in the non-segmented SRM mode.

2.8. Quantitative analysis of the SRM results and data processing

Data were processed with the quantitative software (version B.03.02) and qualitative software (version B.01.03) of Agilent. Peaks were integrated automatically (and verified manually) with the algorithm of the Agilent software after Gaussian smoothing (15 and 5 points for function and Gaussian width, respectively). The relative response uncertainty was set to ± 25% for the ratio of the monitored qualifier transitions.

Endogenous levels of proMMP-9, MMP-9 and TIMP-1were determined in two steps. First the spiked amount of each endogenous amount of each protein manually. Secondly the spiked amount was added to the endogenous amount to calculate the "actual amounts" (see **Table 3**) with the quantitative software. This calibration curve was used to determine endogenous concentrations for each protein using the quantitative software for quantifying unknown samples (see **Table 4**).

2.9. Evaluation of the performance of the SRM assay for the analysis of pro-MMP-9, total MMP-9, and TIMP-1

To assess the repeatability of the SRM assay including sample preparation and LC-MS/MS analysis, a set of 18 samples was prepared and analyzed while following a similar strategy than previously published[38]. To prepare these 18 samples, 6 aliquots (24 µg of total protein each) were ultrafiltered and split in three equal portions before proceeding to protein precipitation with chloroform-methanol, tryptic-digestion, and LC-MS/MS analysis. This strategy allows determining the CV (coefficient of variation) for the ultrafiltration step as well as the CV due to the precipitation, digestion, and LC-MS/MS analysis steps based on the analysis of variance (ANOVA) as described [38, 39].

To assess carry-over during LC-MS/MS analyses, blanks (5% FA / 5% DMSO) were injected at regular intervals. The stability of the target peptides in the autosampler vials was evaluated by injecting the same sample repeatedly throughout the respective analyses.

2.10. Preparation of the immobilized inhibitor material

The inhibitor affinity material was prepared as described previously [15]. A volume of 0.5 mL of NHS-activated Sepharose was washed 3 times with 1 mM HCl at 4º C followed by 3 washes with 500 mM NaCl , 0.2 M phosphate buffer, pH 7.5 (coupling buffer) before reacting with 0.7 mL of a 2 mM

solution of inhibitor (TAPI-2 or ML5 resuspended in coupling buffer) for 2 h at room temperature. The control material was prepared by coupling 0.5 M ethanolamine, pH 8.5 in 0.5 M NaCl for 1 h at room temperature instead of the synthetic inhibitors. Inhibitor and control materials were washed 3 times alternatively with 0.1 M sodium acetate, 0.5 M NaCl, and with the coupling buffer before being stored at 4ºC in PBS supplemented with 20 % ethanol until use.

2.11. Sample preparation for the analysis of enriched, active MMP-9

Control and inhibitor materials were washed before being incubated with the samples of interest in a batch procedure. The washes (3 times for 10 min at room temperature) of the inhibitor or control material (10 µL) were performed with one of the following buffers: PBS (supplemented with 0.1% Tween 20), cell culture medium (supplemented with 0.1% Tween 20), or MMP assay buffer (50 mM Tris, pH 7.4, 0.2 M NaCl, 10 mM $CaCl_2$, 0.005 % (w/v) Brij-35) depending on the samples to be analyzed: BALF, cell culture supernatant, or samples that were treated with APMA, respectively. The samples were then incubated for 30 min at room temperature with the inhibitor or control material. Incubation was followed by three washes of 5 min each (with 100 µL) at room temperature with PBS supplemented with 0.1 % Tween 20 followed by two washes with PBS. The enriched proteins were eluted at room temperature with 20 µL of 20 mM of EDTA (in PBS buffer) in 6 steps of 10 min each.

Eluted fractions were pooled and precipitated with chloroform-methanol for SDS-PAGE analysis, or reduced, alkylated, precipitated with chloroform-methanol and trypsin-digested for SRM analysis. **Figure 1B** presents a general overview of the activity-based enrichment procedure.

2.12. Evaluation of the activity-based enrichment procedure

2.12. a. SDS-PAGE analysis

Flow-through, and eluted fractions of the activity-based enrichment procedure were analyzed by silver-staining of a 12.5 % SDS-PAGE gel to assess the recovery of added MMP-9 CD (0.5 µg) and MMP-12 CD (0.5 µg) and the non-specific binding of MMP-9 and matrix proteins to the inhibitor material. Discontinuous, reducing SDS-PAGE was performed according to Laemmli with 1 mm thick slab gels using a Mini protean III cell assembly (Bio- Rad). Experimental conditions were as follows: stacking gel (1 cm), 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.2% (v/v) TEMED, 125 mM Tris-HCl, pH 6.8; separating gel (6 cm), 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.04% (v/v) TEMED, 375 mM Tris-HCl, pH 8.8; running buffer, 25 mM Tris base, 200 mM glycine, pH 8.3, 0.1% (w/v) SDS. One volume of loading buffer (10 % SDS (w/v), 10 mM DTT, 20% glycerol (v/v), 200 mM Tris-HCl, pH 6.8, 0.05% bromophenol blue (w/v)) was added to four volumes of sample. Samples were heated for 5 min at 95°C and loaded on the gel directly after heating. The run was performed as follows: 10 min at 120 V (stacking) and approximately 50 min at 160 V until migration of the bromophenol blue marker reached the end of the gel. Gels were silver-stained according to [40].

2.12. b. SRM analysis

To assess the feasibility to combine activity-based enrichment with SRM analysis and to assess non-specific binding to the inhibitor and control materials, two calibration curves (spiked with MMP-9 CD and MMP-12 CD, simultaneously) at 0, 0.1 ng/mL, 0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, and 100 ng/mL were prepared for each material.

To demonstrate the activity dependence of the enrichment procedure and to show that only the active enzyme is enriched (and not the pro-forms or the TIMP-inhibited-enzyme complexes) four conditions were tested (in four-fold each). Seven ng/mL of MMP-9 CD, Pro-MMP-9 activated with 1 mM of APMA for 24 h (pro-MMP-9 A), MMP-9 CD inhibited at a 2-fold molar excess of TIMP-1 for 3 h at 37C (MMP-9 CDI), and pro-MMP-9 added to 1 mL of BALF were treated with the inhibitor material to evaluate enrichment. MMP-12 CD and ^{15}N-MMP-12 CD at 7 ng/mL were used as positive controls. Samples were analyzed by LC-MS/MS in the SRM mode with stable-isotope labeled peptides as internal standards.

2.13. Activation of pro-MMP-9 in 16HBEo-cell culture supernatant

Cell culture supernatant was subjected to buffer exchange into MMP assay buffer (Amicon Ultra 0.5 mL centrifugal filter, 10 kDa cut-off) and concentrated ~50 times. For the activation process two conditions were tested: direct activation with 1 mM APMA for 24 h at 37°C, or activation with 1 mM APMA (24 h at 37°C) after incubating the samples with ~200 ng (for 1 mL of original cell culture supernatant) of ^{15}N-MMP-12 CD for 1 h at 37°C to block TIMPs.

2.14. Zymography

Cell culture supernatant (~1 mL), concentrated and activated as described above was incubated at 37°C for 15 min in non-reducing Laemmli buffer (0.4 M Tris-HCl, pH 6.8, 5 % SDS (w/v), 20 %glycerol (v/v), 0.03% bromophenol blue (w/v)) was analyzed in an 8% SDS-PAGE gel containing co-polymerized gelatin at 1 mg/mL (w/v). Electrophoresis was carried out as described above.

Before staining, the zymography gel was washed 3-times (2.5 h in total) with 2.5 % Triton X-100, followed by 3 washes (2.5 h in total) with MMP assay buffer followed by incubation in assay buffer for 16 h at room temperature. Staining was performed in two steps as described by Kleiner *et al.* [6]. The gel was first incubated in 0.5 % Coomassie blue G-250 in 30 % methanol, 10% acetic acid (3 h at room temperature), followed by destaining with 30 % methanol, 10 % acetiq acid.

2.15. Fluorescent measurements

Activity assays were performed in 96-well plates (Costar-white) on a Fluostar Optima plate reader (BMG, Labtech) with 100 µL of a pooled BALF sample (see above). MMP-9 CD, MMP-9 CD inhibited by TIMP-1 (at a 2-fold molar ratio for 3 h at 37°C), pro-MMP-9, or pro-MMP-9 activated by

APMA (24 h at 37°C) were spiked at 10 ng/100 µL and measurements were performed in triplicate. Enzyme activity was measured by following cleavage of the pro-fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 at 2µM for 15 min (λ_{ex}, λ_{em} = 327, 420 nm, respectively).

3. Results

3.1. SRM analysis of pro-MMP-9, total MMP-9, and TIMP-1

3.1. a. Selection of signature peptides

The choice of signature peptides is a crucial step in developing a sensitive, specific, and reproducible SRM assay for the absolute quantification of pro-MMP-9, total MMP-9, and TIMP-1. The following criteria were applied for the selection of signature peptides. The peptides have a minimum length of 6 amino acids, do not include M or C residues which are susceptible to oxidation and do not contain missed cleavages. The *in silico* selected peptides were submitted to a BLASTP search against the UniProt Knowledgebase (*Homo sapiens*) consisting of the UniProtKB/Swiss-Prot Release 2012_05 of 16-May-2012 (20232 entries) and of the UniProtKB/TrEMBL Release 2012_05 of 16-May-2012 (110822 entries) to ensure that they do not occur in any other human protein. Finally, the corresponding purified proteins of interest were trypsin-digested and analyzed by LC-MS/MS to evaluate the response of the candidate peptides experimentally.

In a previous study, the signature peptides AVIDDAFAR and AFALWSAVTPLTFTR belonging to the catalytic domain of MMP-9 have been successfully used to quantify the protease in BALF at the low ng/mL level (see **Table 1**) [34]. Quantification of total MMP-9 was based on AVIDDAFAR, which gave a better response in terms of linearity and sensitivity while AFALWSAVTPLTFTR was monitored for confirmatory evidence.

The number of suitable signature peptides from TIMP-1 is limited. TIMP-1 is a relatively small protein (~23 kDa) with a high number of M and C residues. Three candidate peptides were selected from an *in silico* tryptic digest of TIMP-1: SEEFLIAGK, GFQALGDAADIR, and FVGTPEVNQTTLYQR. Analysis of trypsin-digested TIMP-1 by LC-MS/MS showed a strong response for SEEFLIAGK and GFQALGDAADIR whereas FVGTPEVNQTTLYQR was not detected. SEEFLIAGK and GFQALGDAADIR proved to be specific for TIMP-1 when compared against the UniProt Knowledgebase (*Homo sapiens*) via a BLASTP search. SRM transitions for SEEFLIAGK and GFQALGDAADIR were optimized as described under Material and Methods and lead to the final SRM assay parameters summarized in **Table 1**.

Peptide sequence	Targeted protein	Parent ion (m/z)	FR (V)	Product ion (m/z)	CE (V)
AVIDDAFAR	total MMP-9 or active MMP-9	489.3 (+2)	210	807.4 (y7; +1)	12
				694.3 (y6;+1)	12
				579.3 (y5;+1)	20
				404.2 (y7;+2)	8
AVIDDAFA**R**		494.3 (+2)		817.4 (y7; +1)	12
				704.3 (y6;+1)	12
				589.3 (y5;+1)	20
				409.2 (y7;+2)	8
AFALWSAVTPLTFTR	total MMP-9 or active MMP-9	841 (+2)	220	1092.6 (y10;+1)	33
				219.1 (b2;+1)	41
				290.1 (b3;+1)	49
AFALWSAVTPLTFT**R**		846 (+2)		1102.6 (y10;+1)	33
				219.1 (b2;+1)	41
				290.1 (b3;+1)	49
QLSLPETGELDSATLK	pro-MMP-9	851.5 (+2)	300	1260.6 (y12; +1)	28
				630.8 (y12; +2)	28
				519.3 (y5; +1)	38
				329.2 (b3; +1)	34
QLSLPETGELDSATL**K**		855.5 (+2)		1268.6 (y12; +1)	28
				634.8 (y12; +2)	28
				527.3 (y5; +1)	38
				329.2 (b3; +1)	34
*QLSLPETGELDSATLK	pro-MMP-9	842.9 (+2)	200	1460.7 (y14; +1)	24
				1260.6 (y12; +1)	18
				630.8 (y7; +2)	18
*QLSLPETGELDSATL**K**		846.9 (+2)		1468.7 (y14; +1)	24
				1268.6 (y7; +1)	18
				634.8 (y7; +1)	18
QLAEEYLYR	pro-MMP-9	592.8	220	943.5 (y7; +1)	22
				743.4 (y5; +1)	18
				614.3 (y4; +1)	24
				338.2 (y2; +1)	16
QLAEEYLY**R**		597.8		953.5 (y7; +1)	22
				753.4 (y5; +1)	18
				624.3 (y4; +1)	24
				348.2 (y2; +1)	16
PyroELAAEYLYR	pro-MMP-9	584.3	200	743.4 (y5; +1)	16
				614.3 (y7; +1)	14
				451.3 (y7; +1)	14
				338.2 (y7; +1)	12
PyroELAAEYLY**R**		589.3		753.4 (y7; +1)	16
				624.3 (y7; +1)	14
				461.3 (y7; +1)	14
				348.2 (y7; +1))	12

Table 1 (continued)

Peptide sequence	Targeted protein	Parent ion (m/z)	FR (V)	Product ion (m/z)	CE (V)
SEEFLIAGK	TIMP-1	497.3	110	777.5 (y7; +1)	12
				648.4 (y6; +1)	16
				388.3 (y4; +1)	14
				275.2 (y3; +1)	22
SEEFLIAG**K**		501.3		785.5 (y7; +1)	12
				656.4 (y6; +1)	16
				396.3 (y4; +1)	14
				283.2 (y3; +1)	22
GFQALGDAADIR	TIMP-1	617.3	150	830.4 (y8; +1)	18
				717.4 (y7; +1)	16
				517.3 (b5; +1)	10
				404.2 (b4; +1)	14
GFQALGDADDIR		622.3		840.4 (y7; +1)	18
				727.4 (y7; +1)	16
				517.3 (b5; +1)	10
				404.2 (b4; +1)	14
YVDINTFR	MMP-12	514.3	210	765.4 (y6; +1)	16
				650.4 (y5; +1)	20
				263.1 (b2; +1)	12
YVDINTFR		520.3		775.4 (y6; +1)	16
				659.3 (y5; +1)	20
				265.1 (b2; +1)	12

Table 1. Optimized parameters for pro-MMP-9, total MMP-9 and TIMP-1 SRM assays. Underlined product ions were used for quantification. Peptides labeled with ^{15}N and ^{13}C at the C-terminal R/K (shown in bold) were chosen as internal standards (IS). A glutamine (Q) residue located at the N-terminus of a peptide might be subject to intramolecular rearrangement leading to a pyro-glutamic acid (pyro-E). Transitions of the modified pyro-E-QLAEEYLYR and pyro-E-QLSLPETGELDSATLK were also optimized and monitored during the analyses. Peptides labeled with ^{15}N and ^{13}C at the C-terminal R or K or at every N-atom through metabolic labeling of MMP-12 were chosen as internal standards (shown in bold-underlined). FR: fragmentor voltage. CE: collision energy.

Signature peptides belonging to the pro-domain of MMP-9 were chosen to determine the molar ratio between pro-MMP-9 and total MMP-9, which should be 1 if no activation occurred. The pro-domain of MMP-9 contains 73 amino-acids (residues 20-93 of the sequence given in P14780) and led to 4 predicted tryptic peptides that fulfilled our criteria: QLAEEYLYR, SLGPALLLLQ, QSTLVLFGPGDLR and QLSLPETGELDSATLK. QLSLPETGELDSATLK gave the highest response upon LC-MS/MS analysis and was used for quantification, while QLAEEYLYR was followed by SRM to provide confirmatory evidence of the presence of pro-MMP-9. The optimized SRM conditions for these peptides are given in **Table 1**.

When selecting signature peptides for the pro-domain of MMP-9 we also took the enzymatic and non-enzymatic processes and the reported cleavage sites into account. Pro-MMP-9 is first cleaved, by interstitial collagenase (MMP-1), gelatinase A, stromelysin-1, and collagenase-3, at Q_{40}-M_{41} followed by a second cleavage at R_{87}-F_{88} removing the cysteine switch and leading to active MMP-9.[41] Stromelysin-1 results in a further slow degradation of gelatinase B by cleavage at P_{428}-E_{429}.

Mercuric compounds such as APMA and $HgCl_2$ activate MMP-9 through a multi-step process with a final cleavage site at M_{75} responsible for activation of the enzyme [42]. Nagase *et al.* have shown that mercuric compounds are responsible for a conformational change of pro-MMP-9 but that removal of the pro-domain is an autocatalytic process [43]. We therefore based quantification of pro-MMP-9 on the peptide Q_{58}LSLPETGELDSATLK$_{73}$, which is removed according to all described cleavage events leading to active MMP-9.

A glutamine (Q) residue at the N-terminus of a peptide might be subject to intramolecular rearrangement leading to pyro-glutamic acid (pyro-E). We therefore optimized transitions also for pyro-E-containing QLAEEYLYR as well as for pyro-E-containing QLSLPETGELDSATLK (see Material and Methods and **Table 1**) and monitored these transitions in all assays but there was no indication of pyro-E-containing peptides. Synthetic peptides labeled with ^{15}N and ^{13}C at the C-terminal R/K residues were used as internal standards (IS) and subjected to collision-induced dissociation under identical conditions (see **Table 1** for SRM conditions).

The final multiplexed SRM assay covering 6 peptides and 23 transitions was designed to assess the activity status of MMP-9 in cell culture supernatant as well as the amount of TIMP-1, the major endogenous inhibitor. AVIDDAFAR and AFALWSAVTPLTFTR were used to quantify total MMP-9, while QLAEEYLYR and QLSLPETGELDSATLK provided quantitative data about the amount of pro-MMP-9. The quantification of TIMP-1 was based on SEEFLIAGK and GFQALGDAADIR. The molar ratio between pro-MMP-9 and total MMP-9 provides a measure of the activation level of pro-MMP-9 in relation to the amount of TIMP-1 all in a single LC-MS/MS analysis.

3.1. b. Method development and assay performance

In order to quantify total MMP-9, pro-MMP-9 and TIMP-1 in cell culture supernatant, sample preparation is a critical issue (see **Figure 1** for a schematic overview). To assess the repeatability of the entire assay including sample preparation and LC-MS/MS, we calculated the Relative Standard Deviation (RSD) based on the measured peak area of the quantifier ion of each signature peptide relative to the added internal standard. Quantification of total MMP-9 and pro-MMP-9 had an RSD of 15.4% and 13.4% based on AVIDDAFAR and QLAAEYLYR, respectively (**Table 2**). AFALWSAVTPLTFTR, which was used as confirmatory evidence for total MMP-9, had an RSD of 15.7 % and QLSLPETGELDSATLK, which was used as confirmatory evidence for pro-MMP-9, had an RSD of 15.6% (see **Table 2**). TIMP-1 quantification showed RSDs of 20.2% and 20.3% based on the signature peptides SEEFLIAGK and GFQALGDADDIR, respectively (see **Table 2**).

An ultrafiltration step was added to the previously used sample preparation scheme [34] to concentrate the cell culture supernatant and to exchange the buffer for subsequent DNA/RNA digestion, which was needed to reduce overall viscosity. Since ultrafiltration may lead to protein loss due to adsorption to the membrane, the variability of the ultrafiltration step relative to the other downstream sample preparation steps was assessed by preparing 18 samples according to an experimental design (see Material and Methods). Six aliquots were ultrafiltered and then split

into three equal portions prior to protein reduction and alkylation, precipitation by chloroform-methanol, tryptic-digestion, and LC-MS/MS analysis. ANOVA was used to determine the contribution of the ultrafiltration step and the rest of the procedure to the overall variability. The coefficients of variation (CV) for MMP-9 and TIMP-1-related peptides due to the ultrafiltration step ranged from 6.5 – 10.3 % while the contribution of the 3 ensuing steps (chloroform-methanol precipitation, digestion with trypsin, and analysis by LC-MS/MS) ranged from 8.3% - 15.9% (CV) (see **Table 2**). This indicates that both parts of the procedure contribute to method imprecision, but that the impact of the ultrafiltration step is slightly less pronounced. No carry-over between LC-MS/MS runs was detected when running 4 blanks (5%FA / 5%DMSO) at regular intervals.

3.1. c. Removal of the pro-domain after enzyme activation

Assessing the activation status of MMP-9 based on the molar ratio between signature peptides that are derived from the pro-domain relative to those that quantify the total amount of MMP-9 (catalytic domain-derived signature peptides) is only valid if the pro-domain is efficiently removed prior to trypsin digestion. There are two steps in the sample preparation procedure that are likely suited to separate the pro-domain, comprising amino acids 20-93 of pro-MMP-9 (P14780), from the processed enzyme, notably ultrafiltration at 10 kDa (the pro-domain has a molecular weight of 8334 Da) and chloroform-methanol precipitation. **Figure 2** shows that the pro-domain is completely removed during sample preparation (compare panels A3 - C3) while active MMP-9 is retained (see panels A1 – C1).

It is necessary to block TIMP-1 with an excess MMP-12 (catalytic domain) to achieve full activation (compare for example panels A3 – C3 in **Figure 2;** see also **Figure 7** for the corresponding zymography results). This is in agreement with previous studies of Nagase *et al*. that showed that treatment by APMA is not an activator of pro-MMP-9 when it is complexed with TIMP-1. Interaction of TIMP-1 with pro-MMP-9 occurs through the C-termini of both proteins and prevents the activation of pro-MMP-9 treated by APMA. Nagase *et al.* showed, however, that pro-MMP-9 may be activated if the pro-MMP-9/TIMP-1 complex is incubated with a large excess of MMP-3, since MMP-3 blocks the N-terminus of TIMP-1 before processing pro-MMP-9. This was achieved with an excess of MMP-12 (catalytic domain) in our case.

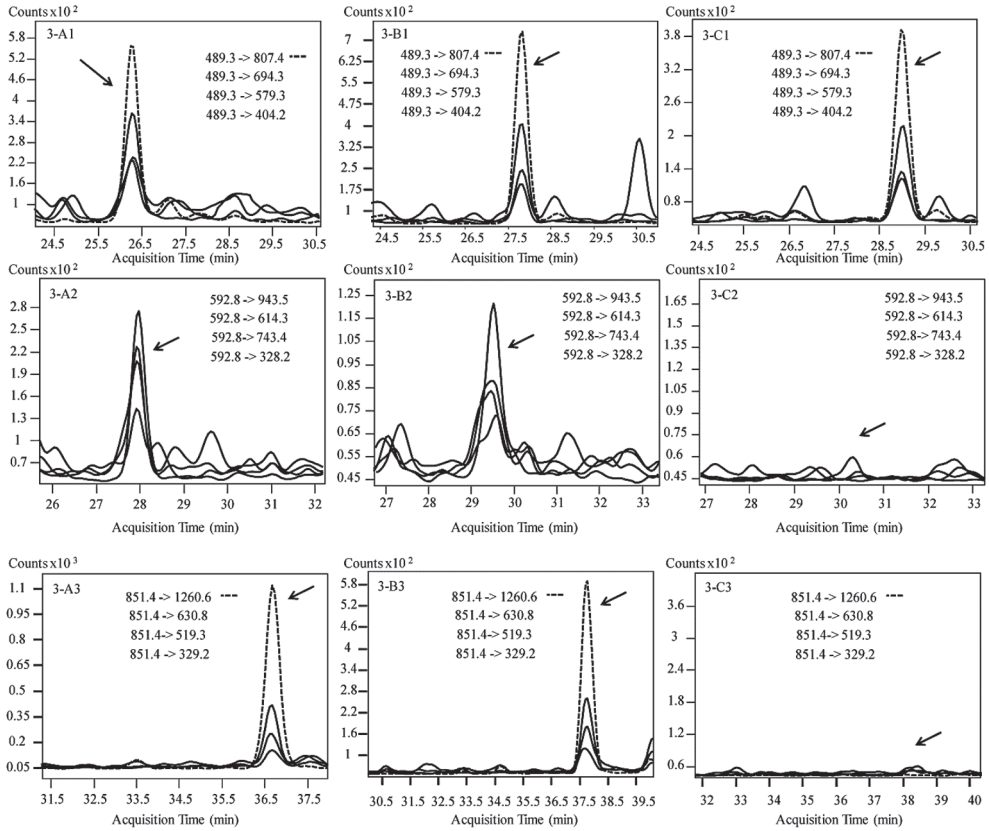

Figure 2. SRM analysis of endogenous MMP-9 from a pool of 16HBE0- cell culture supernatant. Extracted ion chromatograms of the transitions for the signature peptide AVIDDAFAR (489.3→404.2/579.3/694.3/807.4-A1/B1/C1 indicated by an arrow) are compared to signature peptides belonging to the pro-domain: QLAEEYLYR (A2/B2/C2) and QLSLPETGELDSATLK (A3/B3/C3). (A), direct analysis after APMA treatment; (B) direct analysis after APMA treatment following blocking the N-terminus of TIMP-1 by pre-incubation with MMP-12; (C) conditions as under (B) followed by analysis after removal of the pro-domain during sample preparation (see **Figure 1**, left panel). Activation of pro-MMP-9 was confirmed by zymography (see **Figure 7**, **lane 3**).

3.1. d. Absolute quantification of pro-MMP-9, total MMP-9 and total TIMP-1

Quantification of pro-MMP-9, total MMP-9, and TIMP-1 in cell culture supernatant of 16HBEo- cells was based on 6-point external calibration curves (see **Table 3**). Based on previous experiments we decided to calibrate the method between 0.5 - 6.5 ng/mL to cover the expected concentration range of proMMP-9 in 16HBEo- cell culture supernatant. The measured bias ranged from – 4.5 to 29.6% and from -73 to 4% for the signature peptides AVIDDAFAR and QLSLPETGELDSATLK, respectively (**Table 3**). The measurements of TIMP-1 showed a bias of -28.5 to 38.6% over a concentration range of 6.7 – 66.7 ng/mL based on signature peptide GFQALGDADDIR (**Table 3**).

pro-MMP-9 concentration (ng/mL)	Actual Total MMP-9 concentration based on AVIDDAFAR (ng/mL)*	Measured total MMP-9 concentration based on AVIDDAFAR (ng/mL)	Bias (%) Total MMP-9 based on AVIDDAFAR	Actual pro-MMP-9 concentration based on QLSLPETGELDSATLK (ng/mL)**	Measured pro-MMP-9 concentration based on QLSLPETGELDSATLK (ng/mL)	Bias (%) pro-MMP-9 based on QLSLPETGELDSATLK
0	0.60	NA	NA	0.47**	NA	NA
0.5	1.10	1.05	-4.5	0.97	0.26	-73.2
1.5	2.10	1.90	-9.5	1.97	1.57	-20.3
2.5	3.10	3.26	5.2	2.97	3.09	4.0
4.5	5.10	3.59	-29.6	4.97	3.41	-31.4
6.5	7.10	6.04	-14.9	6.97	7.19	3.2

TIMP-1 concentration (ng/mL)	Actual TIMP-1 concentration (ng/mL)***	Measured TIMP-1 concentration based on GFQALGDAADIR (ng/mL)	Bias (%) of TIMP-1 concentration based on GFQALGDAADIR
0	6.7	NA	NA
10	16.7	18.8	12.6
20	26.7	37.0	38.6
30	36.7	26.1	-28.9
40	46.7	62.2	33.2
60	66.7	72.0	7.9

Table 3: Absolute quantification of pro-MMP-9, total MMP-9 and TIMP-1 based on the signature peptides QLSLPETGELDSATLK, AVIDDAFAR, GFQALGDAADIR, respectively (see **Table 1** for the monitored transitions). Calibration curves were prepared in duplicate with 6 calibrant points (see Material and Methods). The biological matrix was a pool of cell culture supernatants from all analysed CSE and CTR samples. Endogenous levels of the targeted proteins were determined from the calibration curves to 0.6 ng/mL pro-MMP-9, and 6.7 ng/mL TIMP-1. Endogenous levels were added to the spiked amounts of the respective target protein to prepare the calibration curve (see Material and Methods). NA: not applicable

Cell passages	Total protein concentration (µg/mL)	Measured total MMP-9 concentration based on AVIDDAFAR (ng/mL)	Measured Total MMP-9 concentration based on AVIDDAFAR Pm	Measured TIMP-1 concentration based on GFQALGDAADIR (ng/mL)	Measured TIMP-1 concentration based on GFQALGDAADIR pM	Measured pro-MMP-9 concentration based on QLSLPETGELDSATLK (ng/mL)	Measured pro-MMP-9 concentration based on QLSLPETGELDSATLK pM
CSE 60	23.4	0.36	4.00	4.24	184.00	1.21	15.87
CSE 70	12.4	0.79	8.77	5.73	248.66	1.37	17.96
CTR 60	23.1	1.20	13.32	25.92	1124.66	7.70	100.96
CTR 70	15.4	0.97	10.76	15.57	675.58	2.74	35.93

Table 4. SRM analysis of total MMP-9, pro-MMP-9, and TIMP-1 (see **Table 1** for the monitored transitions) in the supernatant of 16HBEo- cells that were exposed to cigarette smoke extract (CSE) or to buffer (CTR). Two different cell passages (60, 70, generations) were analysed to evaluate the effect of 10% CSE for 24 h in comparison to control (CTR)

Control cells and cells that were exposed to cigarette smoke extract (CSE) were studied at different cell passages (CSE/CTR 60, CSE/CTR 70) to assess the effect of CSE on the activity status of MMP-9 and the ratio to its main inhibitor TIMP-1. **Table 4** shows a summary of the obtained results. The concentration of pro-MMP-9 based on signature peptide QLSLPETGELDSATLK is in every sample slightly higher than the measured total MMP-9 based on AVIDDAFAR indicating that accuracy of this assay requires further improvement. Despite this limitation, the results indicate that there is no-processing of pro-MMP-9 within the 16HBEo- cell supernatant independently of whether the cells are exposed to CSE or not. These results agree with SRM measurements after activity-dependent enrichment on immobilized inhibitors, where no free active MMP-9 was detected (see **Figure 6**). Our analyses showed further that there is always a significant molar excess of TIMP-1 over MMP-9, but that the molar ratio decreases from 74-fold to 49-fold ($p = 0.025$) upon exposure of the cells to CSE. This is due to a significant decrease in TIMP-1 concentration (see **Table 4**).

3.2. Activity-based enrichment of MMP-9

3.2. a. Method development and assay performance in BALF

Since determination of proMMP-9 activation based on the molar ratio between signature peptides that were derived from the pro-domain relative to those that were derived from the catalytic domain proved to be unreliable at this stage, we combined the SRM assay with activity-dependent enrichment of active MMP-9 to see whether small amounts of uninhibited, active MMP-9 were present. We showed in previous work that spiked recombinant catalytic domains of a range of MMPs (MMP-1, MMP-7, MMP-8, MMP-10, MMP-12, and MMP-13) can be enriched on immobilized inhibitor beads in an activity-dependent manner. Recovery was generally high (above 90 %) in assay buffer. Freije *et al.* demonstrated furthermore the enrichment of active MMPs from synovial fluid of a rheumatoid arthritis patient by subsequent gelatin zymography [26].

The feasibility to combine the SRM assay with activity-based enrichment of MMP-9 was first assessed by determining non-specific protein binding to the Sepharose carrier material by preparing two chromatographic materials. Active ester moieties (N-hydroxysuccinimide (NHS)) on the Sepharose material were either used for immobilization of the synthetic inhibitor TAPI-2 (inhibitor material) or reacted with ethanolamine (control material) [15]. Binding, washing and elution conditions were optimized by spiking the catalytic domain of MMP-9 into BALF to mimic a highly complex biological matrix and subjecting the samples to the inhibitor and the control materials. Flow-through, wash and elution fractions were analyzed by SDS-PAGE after silver staining (**Figure 3**). Three major drawbacks were apparent when performing all steps in the activity assay buffer (50 mM Tris, pH 7.4, 0.2 M NaCl, 10 mM $CaCl_2$, 0.005 % (w/v) Brij-35). First there was significant non-specific binding of BALF-derived proteins to the control material (**Figure 3A, lanes 4-8**) and to the inhibitor material (**Figure 3A, lanes 10-14**). Second auto-degradation products of the spiked MMP-9 catalytic domain (indicated by an arrow in lane 2 of **Figure 3A**) were present in the eluted fractions from the control and inhibitor materials (**Figure 3A, lanes 4-8 and 10-14**, respectively).

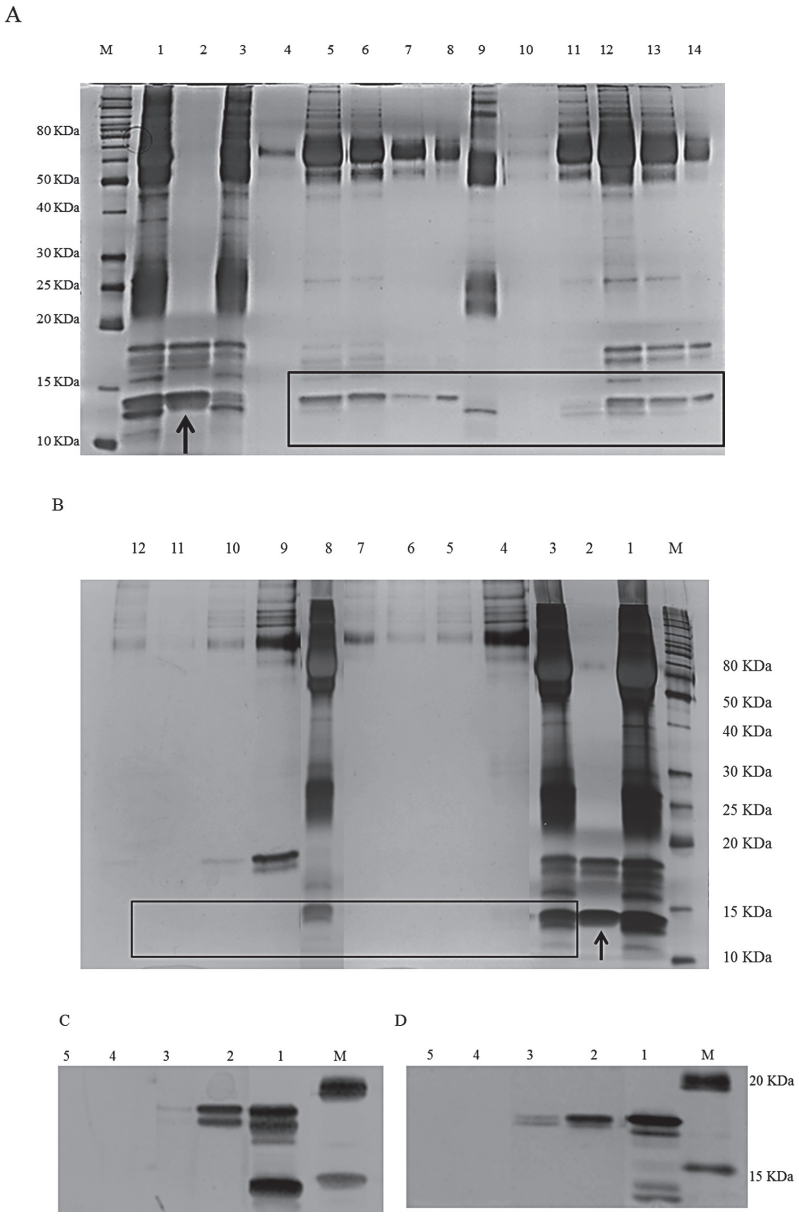

Figure 3.

SDS-PAGE analysis to assess the performance of the activity-based enrichment procedure for MMP-9. MMP-9 catalytic domain (CD) was spiked at 0.5 µg/mL into 1 mL of BALF and activity-based enrichment was performed with MMP assay buffer (see Materials and methods) (**A**) or with PBS containing 0.1% Tween 20 (**B**). Non-bound (flow through) and eluted fractions were analyzed by 12.5% SDS-PAGE and silver staining to evaluate the non-specific binding as well as the recovery of the spiked enzyme. The optimized protocol (performed with PBS containing 0.1% Tween 20) resulted in high recovery of spiked MMP-9, low non-specific binding to the Sepharose material, and complete elution in the presence of 20 mM EDTA.

A. M: Marker; 1: Original BALF sample (spiked with MMP-9 CD); 2: MMP-9 CD positive control; 3-8: experiments performed with the ethanolamine-derivatized, NHS-activated Sepharose (control material); 3: non-bound fraction; 4-7: elution with 20mM EDTA; 8: protein that remained on the beads after elution with EDTA (elution by boiling the beads in Laemmli buffer); 9-14: experiments performed with the TAPI-2 inhibitor material; 9: non-bound fraction; 10-13: elution with 20mM EDTA; 14: protein that remained on the beads after elution with EDTA (elution by boiling the beads in Laemmli buffer).

B. M: Marker; 1: Original BALF sample (spiked with MMP-9 CD); 2: MMP-9 CD positive control; 3-8: experiments performed with the ethanolamine-derivatized, NHS-activated Sepharose (control material); 3: non-bound fraction; 4-7: elution with 20mM EDTA; 8: protein that remained on the beads after elution with EDTA (elution by boiling the beads in Laemmli buffer); 9-14: experiments performed with the TAPI-2 inhibitor material; 9: non-bound fraction; 10-13: elution with 20mM EDTA; 14: protein that remained on the beads after elution with EDTA (elution by boiling the beads in Laemmli buffer).

Auto-degradation fragments from the commercially available rec. MMP-9 CD, are indicated by arrows. While these fragments are enriched by the control and inhibitor materials when using MMP assay buffer due to non-specific binding (panel A, boxed area), they are not captured by the same materials when using PBS containing 0.1% Tween 20 (panel B, boxed area).

SDS-PAGE analysis to assess the performance of the activity-based enrichment procedure for MMP-9. MMP-9 catalytic domain (CD) was spiked at 0.5 µg/mL into 1 mL of 16HBEo-- cell's supernatant. and activity-based enrichment was performed with PBS containing 0.1% Tween 20

C. Eluted fractions were analyzed by 12.5% SDS-PAGE and silver staining to evaluate the non-specific binding as well as the recovery of the spiked enzyme. The optimized protocol resulted in high recovery of spiked MMP-9, low non-specific binding to the Sepharose material, and complete elution in the presence of 20 mM EDTA.

D. To demonstrate that the described methodology is not MMP-9-specific, MMP-12 CD was spiked at 0.5 µg/mL ng/mL into 1 mL of 16HBEo-- cell's supernatant followed by activity-based enrichment. Eluted fractions were analyzed to evaluate the non-specific binding as well as the recovery of the spiked enzyme.

For panels C and D; M: Marker; 1: positive controls (MMP-9 CD and MMP-12 CD); 2-3: experiments performed with the inhibitor material; 3: elution with 20mM EDTA; 4: protein that remained on the beads after elution with EDTA (elution by boiling the beads in Laemmli buffer); 4-5: experiments performed with the inhibitor material; 4: elution with 20mM EDTA; 5: protein that remained on the beads after elution with EDTA (elution by boiling the beads in Laemmli buffer).

Third elution of MMP-9 catalytic domain with EDTA was incomplete, since boiling the beads with SDS-containing sample buffer showed that some protein remained on the inhibitor beads (**Figure 3A, lane 14**).

Supplementing the binding and washing buffer with 0.1% Tween 20 resulted in a significant decrease in non-specific binding to the control (**Figure 3B, lanes 4-7**) and the inhibitor beads (**Figure 3B, lanes 9-12**). Furthermore elution with EDTA from the inhibitor material was complete (**Figure 3B, lanes 9-12**). The final procedure thus consisted of equilibrating the inhibitor beads with PBS + 0.1% Tween 20 and washing the beads 3 times (with 10 column volumes) with the same buffer. Removal of Tween 20 from the affinity-chromatography material was performed by two washes with PBS (10 column volumes) before eluting the enriched protein with 20 mM EDTA in PBS.

3.2. b. Calibration curves

While SDS-PAGE analysis of the optimized activity-based enrichment procedure applied to BALF did not show any non-specific binding of MMP-9 catalytic domain to the control material, an analytical method of higher sensitivity, such as SRM analysis, might still reveal some non-specific

binding. Calibration curves of the combined methodology were thus established for the control and for the inhibitor materials to assess whether the activity-based enrichment procedure is suitable for quantitative work. To show that the proposed methodology is not limited to MMP-9, we added MMP-12 catalytic domain into 1 mL of BALF at 0, 0.1, 0.5, 1, 5, 10, 40 and 100 ng/mL and analyzed the mixtures by SRM (see Table 1). Both analyses resulted in linear calibration curves (MMP-9: y = 67.6 x + 411.15; R^2=0.96; MMP-12: y = 48.25 x + 350; R^2=0.93; **Figure 4 A and B**).

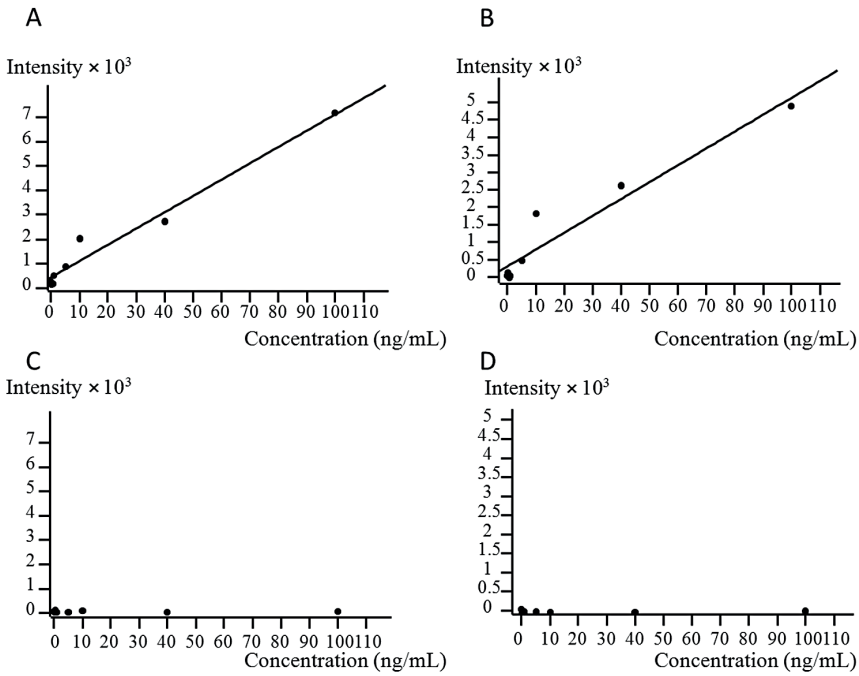

Figure 4.

A. Calibration curve after activity based enrichment with an immobilized broad-spectrum MMP inhibitor [20] of MMP-9 CD spiked into 1 mL of BALF at: 0, 0.1 ng/mL, 0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 40 ng/mL, 100 ng/mL; y= 67.6x + 411.15, R^2=0.96. The calibration curve was based on AVIDDAFAR (quantifier ion, y7: 807.4 (+1)).

B. Calibration curve after activity based enrichment with ML-5 material of MMP-12 CD spiked in 1 mL of BALF at: 0, 0.1 ng/mL, 0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 40 ng/mL, 100 ng/mL; y= 48.25x + 350.09, R^2=0.93. The calibration curve was based on YVDINTFR (quantifier ion: y6: 765.4 (+1)).

C/D. Calibration curves were performed in a similar manner as shown in panels A and B with the control material to demonstrate specific enrichment of spiked MMP-9 CD (**C**) and spiked MMP-12 CD (**D**). While detection of the spiked enzymes was possible based on co-elution with the stable-isotope-labeled internal standard peptides, peak areas did not correlate with the amount of added enzymes; y= 0.058 x+ 63.10, R^2=0.0046 (**C**) and y= -0.00176 x + 47.007, R^2=0.0004932 (**D**) for MMP-9 and MMP-12, respectively.

The calibration curves with the control material showed very low but detectable non-specific binding of spiked MMP-9 and MMP-12 catalytic domains (**Figure 4 C and D**). Background binding was, however, not concentration dependent (R^2=0.0046 and 0.00049 for MMP-9 and MMP-12,

respectively). The limit of linearity was estimated to be 1 ng/mL and 5 ng/mL for MMP-9 and MMP-12, respectively. Below these levels, the signature peptides AVIDDAFAR (for MMP-9) and YVDINTFR (for MMP-12) were still detected but the measured peak area of the quantifier ion was similar to the one detected with the control material. Non-specific binding to the Sepharose material was thus the main factor limiting sensitivity of the method rather than sensitivity of the LC-MS/MS method itself. Considering that this is a fairly complex sample preparation procedure, we conclude that activity-dependent enrichment can be combined with SRM assays to quantify active MMP-9 and MMP-12 in BALF at the low ng/mL level.

3.2. c. Activity-dependence of enrichment and overall repeatability of the procedure

In order to assure activity-dependence of the procedure, MMP-9 catalytic domain (MMP-9 CD), pro-MMP-9 that was activated by APMA (MMP-9 A), pro-MMP-9 and MMP-9 CD after inhibition by TIMP-1 were spiked into BALF at 7 ng/mL and incubated with the inhibitor material (see Material and Methods). **Figure 5** shows that only MMP-9 CD and MMP-9 A bound to the immobilized inhibitor material and were detected by SRM while inhibited MMP-9 and the inactive pro-enzyme resulted in peaks for the signature peptides that corresponded to background levels due to non-specific binding. Measurements of enzymatic activity using a low-molecular weight pro-fluorescent substrate were in overall agreement with the SRM measurements emphasizing that enrichment was activity-dependent.

The experiments were performed in four-fold to assess the precision of the procedure resulting in an average RSD of 27.2 % for MMP-9 CD and 13.5% for MMP-9 A. The variability for MMP-9 CD is somewhat higher than for the SRM assay alone (see **Table 2**), which is likely due to the additional activity-based enrichment step and the subsequent need to precipitate a few µg of enriched protein with chloroform-methanol.

3.2. d. Method development and assay performance in cell culture supernatant

The activity-based enrichment procedure combined with the SRM assay, as optimized for BALF, was adapted to cell culture supernatant from a human epithelial cell line 16HBEo- in order to study the effect of cigarette smoke extract (CSE) on the MMP-9 – TIMP-1 balance and the activation status of MMP-9.

Since the composition of cell culture supernatant differs significantly from that of BALF, it is important to revisit the non-specific binding of proteins to the inhibitor and the control materials. Non-specific binding was assessed by adding a known amount of MMP-9 CD (0.5 µg) to cell culture supernatant and subjecting it to enrichment on both materials according to the optimized protocol for BALF. The flow-through and elution fractions (see **Figures 3C 3D** for eluted fractions) were analyzed by SDS-PAGE and the inhibitor and control beads were boiled in SDS-containing sample buffer to see whether there were irreversibly bound proteins remaining on the beads after elution.

Figure 3C shows that MMP-9 is enriched by the inhibitor beads and eluted with EDTA. Non-specific protein binding was comparable to that in BALF and both MMP-9 CD and MMP-12 CD

were completely extracted from cell culture supernatant. Importantly the eluted fractions from the control material did not contain a detectable amount of MMP-9 CD. Irreversible binding of MMP-9 to the beads is minor and hardly detectable (see **Figure 3C, lane 3**) indicating that elution with EDTA was almost complete. Comparable results were obtained for MMP-12 CD (**Figure 3D**). However, enriched MMP-12 CD is not completely eluted from the inhibitor material indicating some aggregation of the protein on the beads (**Figure 3D, lane 3**).

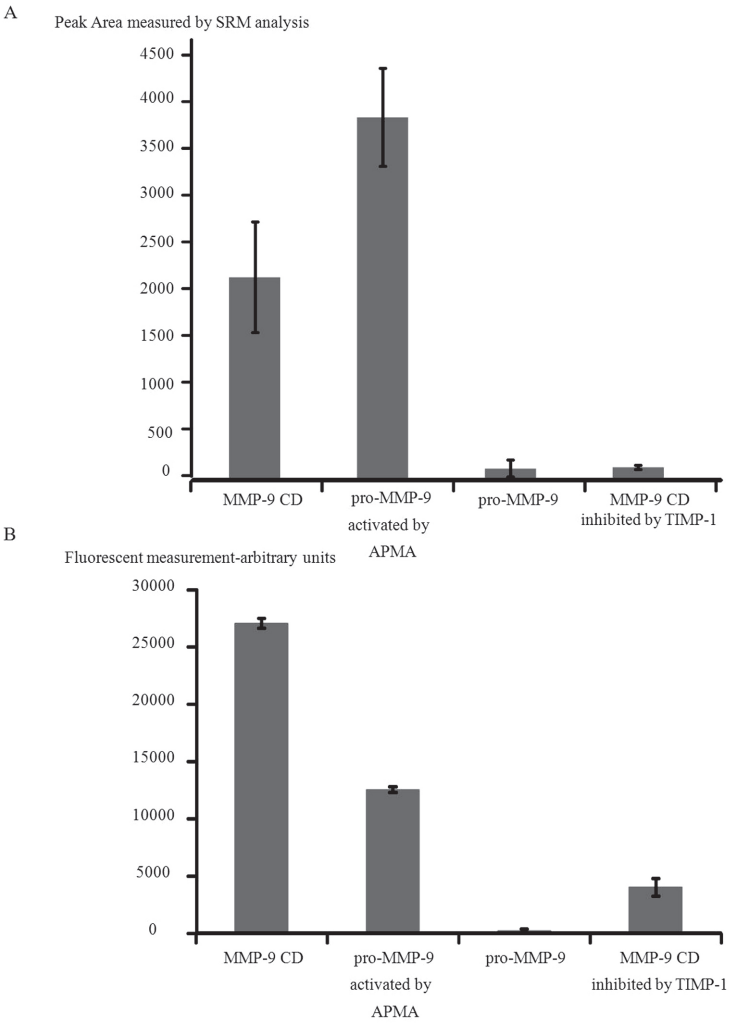

Figure 5. Activity-dependence of the enrichment of MMP-9 on immobilized TAPI-2 beads assessed by SRM analysis (**A**) or conversion of the pro-fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (**B**). Four conditions were tested in 4-fold each at a spiking level of 7 ng/mL in 1 mL of BALF; 1) addition of MMP-9 CD, 2) addition of pro-MMP-9 A (pro-MMP-9 activated with APMA), 3) addition of pro-MMP-9 (non-activated), 4) addition of MMP-9 CDI (MMP-9 CD that was inhibited with TIMP-1). Only active MMP-9 (MMP-9 CD and pro-MMP-9 A) were enriched and detected by the SRM assays.

3.2. e. Application to culture supernatant of 16HBEo-cells exposed to cigarette smoke extract

SRM analysis of cell culture supernatant after exposure to cigarette smoke extract (CSE) or buffer (CTR) showed that MMP-9 was secreted as its pro-form and that the supernatant contained a 73-fold molar excess of TIMP-1 over pro-MMP-9. The molar ratio between the pro-domain-derived signature peptides and the peptides quantifying total MMP-9 indicated that there was little or no activation of pro-MMP-9 under these conditions although variability between different cell culture experiments did not allow drawing conclusions about the levels of activation.

Inhibitor affinity chromatography followed by SRM analysis showed that there was no free, active MMP-9 in cell culture supernatant of either CSE-exposed or control 16HBEo-cells (**Figure 6A, upper panel**) while the stable-isotope-labeled internal standard peptide (**Figure 6A, lower panel**) was clearly detectable.

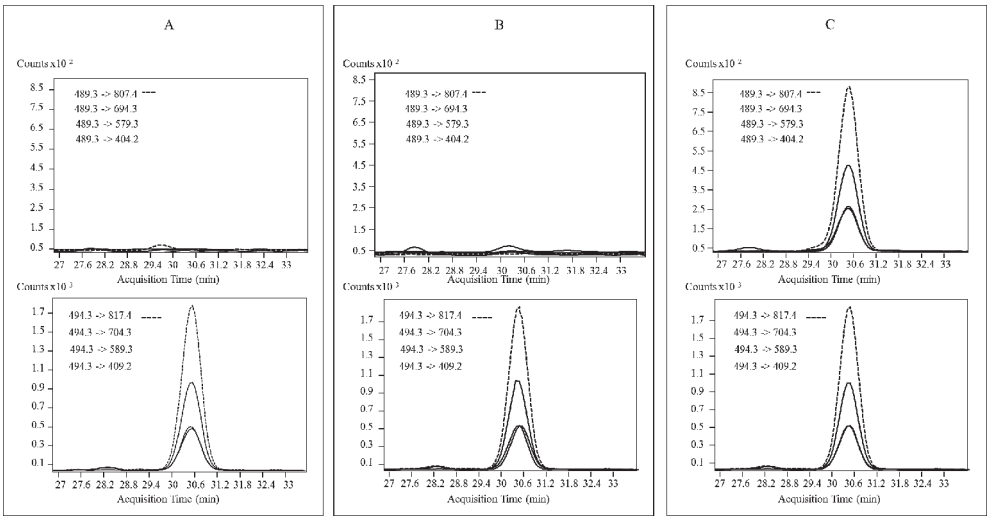

Figure 6. LC-MS/MS analysis (SRM mode) after activity-based enrichment for the study of active or chemically (APMA) activated endogenous MMP-9 in 16HBEo- cell culture supernatant. Activity-based enrichment was performed under three conditions: direct enrichment (**A**), enrichment after APMA treatment (**B**), and enrichment after blocking TIMP-1 with MMP-12 CD prior to APMA treatment (**C**). Extracted ion chromatograms of the transitions (489.3→404.2/579.3/694.3/807.4) for the target peptide AVIDDAFAR (**A1, B1, C1**) and for the corresponding transitions of the stable-isotope-labeled internal standard (IS) peptide (494.3→409.2/589.3/704.3/817.4; **A2, B2, C2**) were monitored. Pretreatment with MMP-12 CD prior to APMA treatment (panel **C**) was the only condition that allowed to detect active MMP-9 after activity-based enrichment based on the monitored transitions and co-elution with the IS peptide. No active MMP-9 was detected after direct enrichment from cell culture supernatant (panel **A**) or following APMA treatment (panel **B**). The transitions 489.3→807.4 and 494.3→817.4, represented as dashed lines, are the quantifier ions.

APMA was used to investigate whether pro-MMP-9 could be activated in cell culture supernatant and thus enriched on the inhibitor material. Interestingly, no active MMP-9 was extracted under these conditions (**Figure 6B, upper panel; the lower panel shows the internal standard peptide**)

indicating that pro-MMP-9, if activated, was captured by the excess TIMP-1 or that the pro-enzyme is already in a complex with TIMP-1, which prevents its activation by APMA as described by Nagase *et al.* [44].

As described in Chapter 3, interaction of TIMP-1 and pro-MMP-9 occurs through the C-termini of both proteins while the extremity of the N-terminus of TIMP-1 is responsible for complexing and inhibiting active MMP-9 [44, 45]. Nagase *et al.* showed that pro-MMP-9 may be activated while in the pro-MMP-9/TIMP-1 complex when incubated with a large excess of MMP-3, since MMP-3 blocks the N-terminus of TIMP-1 before processing and activating pro-MMP-9 [44]. With this consideration in mind, activation of endogenous pro-MMP-9 by APMA was assessed after blocking the N-terminus of TIMP-1 with an excess of MMP-12 CD. The treated samples were incubated with the inhibitor material and the eluted fractions analyzed by SRM leading to a significant amount of free, active MMP-9 that was captured by the inhibitor material (**Figure 6C, upper panel; the lower panel shows the internal standard peptide**).

3.3. Zymography

Analysis of 16HBEo- cell culture supernatant after exposure to cigarette smoke extract (CSE) or buffer (CTR) by direct SRM analysis or by SRM following activity-based enrichment showed secretion of pro-MMP-9 together with a large molar excess of TIMP-1 (ca. 73-fold for CTR and 49-fold for CSE). To compare these results with a widely used method that is based on detecting enzyme activity, the cell culture supernatants were analyzed by gelatin zymography as such and after APMA treatment to activate the gelatinases pro-MMP-9 and pro-MMP-2. APMA treatment was also assessed after blocking the N-terminus of TIMP-1 through reaction with MMP-12 CD.

Zymography showed the presence of two bands in the original samples (**Figure 7, lanes 1 and 4**) which correspond to pro-MMP-9 and pro-MMP-2 according to the molecular weights of ~95 kDa and ~65 kDa, respectively [44-46]. Co-migration of pro-MMP-9 with purified pro-MMP-9 (**Figure 7, lane 7**) supported the assignment. Activation with APMA lead to a characteristic band shift of ~ -10 kDa for pro-MMP-9 in agreement with previously reported results (**Figure 7**, **lanes 2 and 5**) [44-46] whereas pro-MMP-2 was not affected by APMA. This is in agreement with the SRM analyses who indicated that pro-MMP-9 is complexed with TIMP-1, since Nagase *et al*. showed that TIMP-1-complexed pro-MMP-9 is processed to a non-active form with a molecular weight of ~80 kDa after APMA treatment, while TIMP-2-complexed pro-MMP-2 remains unaltered [44-46].

Zymography analysis after APMA treatment and prior inactivation of TIMPs with MMP-12 CD resulted in bands at ~80 kDa and ~60 kDa (**Figure 7**, **lanes 3 and 6)** in agreement with previously published data [45]. Processing of pro-MMP-9 with APMA is a slow phenomenon requiring up to 96 h for completion, while processing of pro-MMP-2 is faster and requires only a few hours to reach completion. Active MMP-2 is, however, totally degraded after 24 h under these conditions [46] and thus no longer visible on the zymogram.

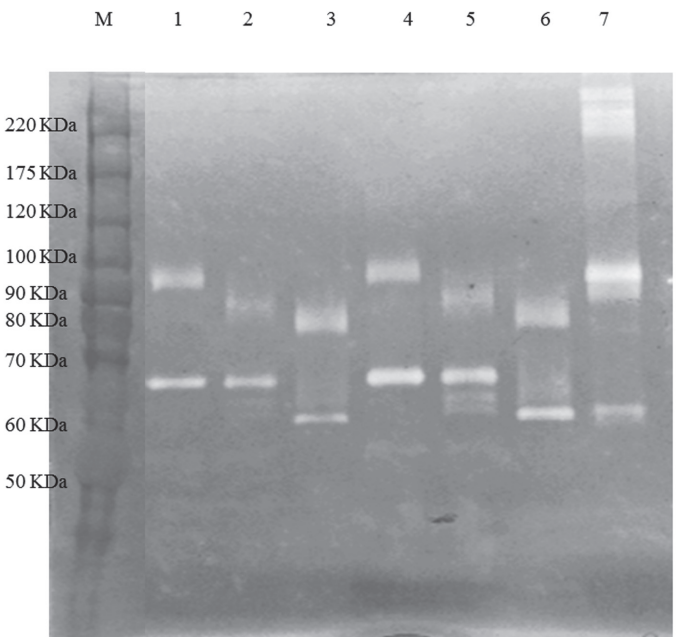

Figure 7. Gelatin zymography of cell culture supernatant from cigarette smoke extract (CSE)-treated and buffer-treated (CTR) 16HBEo- cells. An original volume of 600 µL was concentrated to 20 uL by ultrafiltration at 10 kDa prior to zymography. The activation status of pro-MMP-9 and pro-MMP-2 in the original samples (lanes 1 and 4 for CTR and CSE, respectively) was compared to that of APMA-treated samples (lanes 2 and 5 for CTR and CSE, respectively) or to samples that were treated with APMA after prior inactivation of TIMPs with MMP-12 CD (lanes 3 and 6 for CTR and CSE, respectively). Lane 7 shows the pattern for commercially available purified pro-MMP-9 (0.9 ng, R&D Systems). M: molecular weight marker.

Activity-based enrichment followed by SRM analysis showed that activation of endogenous pro-MMP-9 by APMA was only possible after prior blocking of the N-terminus of TIMP-1 with an excess of MMP-12 CD (**Figure 6C**). This is corroborated by zymography which showed that only this case lead to an active enzyme of ~80 kDa and ~60 kDa (**Figure 7, lanes 3 and 6**) as reported by Nagase *et al.* [45]. Furthermore, the SRM assay with or without prior activity-dependent enrichment showed no endogenous active MMP-9 in agreement with zymography analysis (**Figure 7, lanes 2 and 4**). These results show also that gelatin zymography on its own is not able to discriminate between active and inactive forms of the gelatinases in biological samples unless combined with additional analytical data.

4. Discussion and Conclusions

Assessing the activity status of MMPs in complex biological samples in relation to their endogenous inhibitors is a challenge. However, it is likely that our understanding of the roles of these enzymes in biology and notably disease-related pathology will remain incomplete unless

we can describe the protease-inhibitor system in a comprehensive and quantitative manner. In the past decade, the following main strategies have been explored to tackle this problem; zymography, activity-dependent immunoassays as described by Verheijen *et al.* [7,8], the use of activity-based probes (ABPs) as pioneered by Cravatt *et al.* for serine hydrolases [9,10] and later extended to metalloproteases [11-14] and activity-dependent affinity chromatography [15,17-20,26].

Although promising results were obtained with activity-based probes or activity-dependent affinity chromatography, most of the cited studies are semi-quantitative at best and often do not provide the necessary sensitivity and selectivity that is required to understand the subtle changes in protease activation and inhibition related to biological and pathological mechanisms. The present study builds on this work and describes a novel strategy to assess the activity of MMPs in biological samples. While our work has focused on the analysis of MMP-9 its multiplexing capacity is obvious due to the nature of SRM assays. Our SRM assay provides absolute quantification of pro and active MMP-9 and the main endogenous inhibitor TIMP-1.

Quantifying MMP-9 in the supernatant of 16HBEo- human epithelial cells presents several analytical challenges. The first concerns the choice of the biological matrix in which to establish the calibration curve. We chose to combine aliquots from each cell culture experiment into one pool and to use this as a representative biological matrix for spiking. This ensures that matrix effects will be similar to those observed in the individual samples. A disadvantage is, however, that the proteins of interest (MMP-9 and TIMP-1) are already present in the pooled matrix at approximately the same level as in the samples. It may even occur that a given sample contains a lower concentration of one of the proteins than the pooled matrix that was used to establish the calibration curve. The use of a surrogate matrix that does not contain the analytes of interest might be an alternative but we considered that it would be difficult to mimic the complexity of the cell culture supernatant. To arrive at the actual concentrations for MMP-9 and TIMP-1 in individual samples, we subtracted the amount that is present in the matrix from the measured amount and used a limited standard addition design to assure linearity over the respective range. Bias increased to a maximum of -73% at 0.5 ng/mL, the lowest level for pro-MMP-9 (see **Table 3**) while all other values ranged between -31 and 39%. Although this is outside the range for a validated bioanalytical LC-MS/MS assay according to international guidelines our method is the first activity-dependent analysis of MMPs based on SRM with performance characteristics that are not equaled by other, widely used approaches such as zymography. We realize, however, that further improvements are needed if small levels of enzyme activation shall be reliably quantified, for example, based on molar ratios between signature peptides that are derived from different domains of the enzymes.

Another challenge is the rather complex multistep sample preparation procedure that is needed to achieve concentration sensitivities in the low-medium pM range. Sample preparation can lead to technical variability that may obscure subtle biological differences. One way to compensate for technical variability is to add a stable isotope-labeled internal standard [47]. Since we did not dispose of stable-isotope-labeled pro-MMP-9, MMP-9 catalytic domain or TIMP-1, we added stable-isotope-labeled peptides at the digestion step of the procedure (see **Figure 1**). With this correction, sample

preparation and LC-MS/MS analysis showed an overall repeatability of 15.4% for total MMP-9, 13.4% for pro-MMP-9 and 20.2% for TIMP-1 (see **Table 2**). Interestingly, repeatability of the none-corrected results was slightly better (10.3 %, 11.0 %, and 13.0 % for total MMP-9, pro-MMP-9, and TIMP-1, respectively). Although the reasons for this difference are not clear, one explanation may be that adding the internal standard at such a late stage of the sample preparation procedure does not compensate for variability of the earlier steps and eventually may make matters worse due to a different digestion kinetic and stability during digestion.

A third challenge concerns the quality of commercially available recombinant protein standards. Since pro-MMP-9, used for method calibration, showed some autoactivation (see **Figure 7, lane 7**) it is difficult to measure subtle changes in the balance between pro-MMP-9 and active MMP-9. Our combined strategy of activity-dependent enrichment and SRM has, however, shown that there is no detectable free, active MMP-9 in the cell culture supernatant of 16HBEo- human epithelial cells whether they were exposed to cigarette smoke extract or not. It will be challenging to prepare homogenous standards of metalloproteases due the inherent risk of autoactivation and subsequent autodegradation.

Our activity-dependent enrichment – SRM method reached a sensitivity of approximately 1 ng/mL (50 pM) for MMP-9 in cell culture supernatant and in BALF. It is highly likely that the actual sensitivity is considerably lower, since a significant part of the commercially available rec. MMP-9 catalytic domain was already degraded and thus not captured by the immobilized inhibitor (see **Figure 3D, lane 1**). The major limiting factor to reaching a lower concentration sensitivity appears to be non-specific binding to Sepharose despite the fact that optimization of the binding, washing and elution conditions reduced background binding significantly. There is thus a need for chromatographic materials with less non-specific protein binding. We previously tested a range of other materials, notably HPLC-grade supports, but found Sepharose to have the lowest non-specific binding.

Based on our analyses we conclude that the molar ratio of TIMP-1 to total MMP-9 (essentially pro-MMP-9) in cell culture supernatant of human epithelial cells decreased from about 73-fold to 49-fold upon exposure to cigarette smoke extract (CSE). This indicates that while CSE does affect the inhibitor-protease balance, it is insufficient to inverse it. We are aware that data from cells in culture can only give an indirect view on the *in vivo* situation, but other results in BALF confirm the general view that there is a significant molar excess of inhibitor over protease. It is thus likely that MMP-9 exerts its activity locally and for a limited timespan after activation before being inhibited by TIMP-1. In order to understand the entire MMP-TIMP system, it will be necessary to measure MMPs that are complexed by TIMPs as this will provide information about MMPs that were active in the past. We are currently looking into methodology to capture TIMP-MMP complexes and to determine the MMP content by multiplexed SRM assays in order to complete our picture of the TIMP-MMP system. We strongly believe that such a comprehensive view is needed to gain a better understanding of the role of MMPs and TIMPs in pathological situations and to decipher their role in the many biological mechanisms that these enzymes are implicated in.

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Chapter 5

Summary and perspectives

Matrix metalloproteases (MMPs) are involved in a wide range of biological processes related to extracellular matrix (ECM) remodeling and tissue resorption. The proteolytic activity of MMPs is tightly regulated *in vivo* at multiple levels including gene expression, extracellular and/or intracellular compartmentalization of the enzyme, activation of the proenzyme (zymogen form), and inhibition of the active enzyme by α_2-macroglobulin and tissue inhibitors of metalloproteases (TIMPs). Due to the tight regulation of MMP activity, measurement of active MMPs is more meaningful for the characterization of biological or pathological processes than determination of the overall abundance.

The main focus of this PhD project was the development of analytical techniques for monitoring MMP activity. Photoaffinity-labeling, absolute quantification by mass-spectrometry in the Selected Reaction Monitoring (SRM) mode, and activity-based enrichment techniques were investigated.

Two photoaffinity-labeling probes based on the succinyl hydroxamate motif have been previously described.[1] The two tested probes differ in the position of the photoreactive (trifluoromethylphenyldiazirine) group. Directing the photoactivatable group towards the S1' pocket of MMPs resulted in more effective, family-wide labeling than directing the same photoactivatable group towards the S2' pocket.

Chapter 2 of this thesis presents the labeling study in greater detail. Labeling with the S1'-directed probe resulted in a 1:1 ratio between probe and recombinant MMP-12 CD (catalytic domain). We show furthermore that the labeled protein can be enriched with monomeric avidin beads based on the biotin residue that forms part of the probe. Characterization of the amino acid(s) implied in the covalent bond(s) with the synthetic probes is still under investigation. Despite placing the photoreactive group in the S1' pocket, photolabeling efficiency was rather low (a few percent).

Biological application of the photoaffinity-labeling strategy to monitor MMP activity in biological fluids would require more efficient labeling. Most presently available MMP inhibitors have broad spectrum affinity for all MMPs. Although this may be less suitable for therapeutic intervention, broad spectrum inhibition allows the profiling of protease activity on a family-wide scale. Furthermore, most of the MMP inhibitors show an inhibition in the low nM or even pM range. Thus the main problem of the ABP strategy does not lie in the inhibition pattern but in the low labeling efficiency. Labeling is never complete and the efficiency of labeling varies considerably between different MMPs. An additional problem with photocrosslinking probes is the fact that the crosslinking step is not a quantitative process, and may be influenced by many experimental factors. This limits the use of this technique to semi-quantiative or even only qualitative studies.

Intensive efforts were made for improving the ABP strategy (e.g. chemical synthesis, ABP-enzyme modelling studies). Due to the shortcoming of the labeling efficiency, it is rather difficult to predict the success of this strategy for the study of biological samples. However, one very recent published result shows the value of ABP for detecting endogenous active MMP [2]. The first endogenous active MMP (MMP-12 in BALF from male C57BL/6 mice) has been detected with the use of a phosphinic peptide inhibitor carrying a trifluoromethyldiazirine moiety at the P1' side

chain as well as a tritium atom to improve the detection by radioimaging resulting in an sensitivity of 1 fmol for murine MMP-12. Sensitive, highly reproducible, and low cost assays are required for quantifying low abundant proteins such as MMPs. In recent years, there has been increasing interest in the use of liquid chromatography – tandem mass spectrometry (LC-MS/MS) in the selected reaction monitoring (SRM) mode for protein quantification. The main drawback of this technique, compared to immunoassays, is its lower sensitivity. SRM protein quantification in plasma, without immunodepletion and/or extensive fractionation steps, allows protein detection at the low µg/mL level.

Bronchoalveolar Lavage Fluid (BALF) is, however, a more suitable sample than plasma when studying pulmonary disease, since BALF gives a representative picture of processes occurring in the airways. Nevertheless the characteristics of this matrix for targeted analysis remains unknown. To further assess the role of MMP-9 in pulmonary disease, and notably in the rejection of lung transplants, we established a human MMP-9-specific SRM assay for BALF (**Chapter 3**). This assay does not require antibodies and uses a fully automated microfluidics-based nanoLC-MS/MS system coupled on-line to a triple quadrupole mass spectrometer allowing quantifying MMP-9 at the low ng/mL level. This sensitivity was achieved by optimizing ion transmission in the mass spectrometer, the maximum amount loadable on the HPLC chip, as well as the gradient of the HPLC analysis.

Methanol-chloroform precipitation is a fast, low-cost, highly reproducible step, and thus a key point of the developed assay. Methanol-chloroform precipitation as sample preparation removes the highly abundant phospholipids (known to cause ion suppression effects), concentrate the proteins before their denaturation to unsure an efficient tryptic digestion.

The developed SRM assay was applied to the analysis of BALF samples (5 with and 5 without acute rejection of the transplanted lung). Four out of 10 BALF samples showed an MMP-9 concentration above the LLOQ (2.9 ng/mL). One sample showed an endogenous MMP-9 level slightly below the LLOQ. MMP-9 was detectable in 3 other BALF samples above the LLOD with an SNR between 3 and 10 and co-elution with the internal standard peptide but the ratio between parent ion and fragment ions did not meet the criterion of being within +/- 25% of the expected values. MMP-9 was not detectable in the two other samples.

This shows that sensitivity of the developed assay (**Chapter 3**) must be further increased. Immunoaffinity enrichment and SCX (Strong Cation Exchange) fractionation have been studied for this purpose. Agilent ZORBAX Bio-SCX series II (3.5 µm/225 Å zorbax silica particles coated with sulfonic acid) is the most promising SCX material tested. Neverthelesshis technique, which showed an overall sensitivity improvement of a factor 2.5, was no further investigated due to the time required for the sample preparation. Immunoaffinity enrichment of MMP-9 and MMP-12 spiked in BALF sample has also been tested(immunoprecipitation kit G Dynabeads from Invitrogen (100.07D)) . Even if numerous investigations have been performed for developing an immunoaffinity enrichment (combined to LC-MSMS analysis) no reproducible results were obtained.

Increasing the sensitivity of the developed assay, by immunoaffinity enrichment and by SCX

fractionation, has a major inconvenient : the multiplexing capacity of the SRM assay would no longer be kept. Immuno-depletion of the most abundant proteins would offer the advantage of reducing the sample complexity while keeping all compounds of interest in one set. However this technique has its own problem: co-depletion of the target compounds with the most abundant proteins.

Gelatin chromatography could be another strategy for enriching MMP-9 in biological fluids. This technique is widely used for the purification of all forms of MMP-9 (via the recognition of the gelation by the fibronectin domain of MMP-9). Nevertheless this technique could only be applied to MMPs which have a high gelatin affinity (mainly MMP-9 and MMP-2).

Measuring activity of MMP-9 is more meaningful than measuring the total amount. Innovatives techniques, based on Selected Reaction Monitoring and activity-based enrichment, are described in **Chapter 4**. Peptides of the pro-domain, of the active form and of TIMP-1 are targeted in one single SRM analysis. AVIDDAFAR and AFALWSAVTPLTFTR are used for quantifying total MMP-9, while QLAEEYLYR and QLSLPETGELDSATLK are targeted for quantification of pro-MMP-9. Quantification of TIMP-1 is based on SEEFLIAGK and GFQALGDAADIR.

The multiplex LC-MS/MS method (**Chapter 4**) was applied to study the protease-inhibitor balance in supernatant of human epithelial cells that were exposed to cigarette smoke extract (CSE). Our results showed that there is a considerable molar excess of TIMP-1 over MMP-9 in cell culture supernatant. Secondly, based on the molar ratio between signature peptides from the pro-domain and the catalytic domain even after CSE stimulation no free, active MMP-9 was detectable. Our results show, however, that the molar ratio between TIMP-1 and proMMP-9 decrease significantly when human epithelial cells are exposed to CSE due a lower concentration of TIMP-1.

To determine whether there is a small amount of free, active MMP-9 in the cell culture supernatant after CSE-stimulation or whether all active enzyme is inhibitor-bound, we combined the SRM assays with an activity-dependent affinity enrichment step using immobilized broad-spectrum MMP inhibitors (using a similar strategy than the one published by Freije et *al.*). While active MMP-9 was easily detectable after chemical activation with APMA (4-aminophenylmercuric acetate) and prior blocking the excess of TIMP-1 by pre-incubation with active MMP-12, it was not possible to detect endogenous active MMP-9 after affinity enrichment.

We show the feasibility of assessing the stoichiometry between pro-MMP-9, active MMP-9 and TIMP-1 in a complex biological system by targeted LC-MS/MS in the SRM mode, an approach that may be combined with activity-dependent enrichment techniques. One of the main drawbacks of the developed assays remains the bias of the developed method. Subtle measurement into the CSE case versus the CTR one might need more accurate measurements for assessing clearly the effect of the cigarette smoked extract.

Improvement of the bias of the methods developed into the **Chapter 4** could be easily obtained with the use of labeled protein internal standard. None-corrected repeatability was slightly better (10.3 %, 11.0 %, and 13.0 % for the absolute quantification of total MMP-9, pro-MMP-9, and TIMP-1, respectively) than the corrected one. Although the reasons for this difference are not clear, an

explanation may be that adding the internal standard at such a late stage in the sample preparation procedure does not compensate for variability of the earlier steps.

Application of the activity-based strategy assay to the study of a high number of samples would require automated analysis. Automated integrated system developed by Freije *et al.* [3]could be coupled to the developed SRM assay. The system includes an immobilized inhibitor cartridge [4] (based on peptide-like structures combined with a hydroxamate Zn^{2+}-chelating moiety) for activity-dependent enrichment of the active enzymes, an immobilized trypsin reactor [5] for on-line proteolytic digestion and an LC-MS/MS system (ion-trap for separation and identification of the obtained peptide fragments) might be combined to the developed assays. The automated process was operated by a PROSPEKT-2 automated system (with a Triathlon autosampler). Although this system allows detecting high femtomol levels of enzymes the system lack of robustness mainly caused by the use of the capillary monolithic HPLC column and the in-house modified nano-ESI interface. In the PhD thesis of Theo Klein (http://dissertations.ub.rug.nl/faculties/science/2008/t.klein/) , the performance of the overall system was improved. The monolithic HPLC column was replaced by a chipcube combining an analytical C18 (75 µm ID) with an ESI emitter and a 40 nL C18 trapping column. The robustness of the system was increased; secondly microfluidics device offer the advantage of a higher sensitivity, lower sample consumption, and faster analysis. Another important problem that needs to be tackled for the activity-based and targeted analyses of Matrix Metalloproteases relates to MMPs storage. Indeed, MMP9 (and probably other MMPs) degrade even at -80°C over longer periods of time. One publication gives levels of 65% loss after 2 years at -80°C [6]. The stabilization of MMP-containing samples must therefore be tackled if retrospective sample collections shall be compared. To achieve this it may be advantageous to stabilize active MMPs by adding immobilized inhibitors. Collected samples could be directly incubated with the material used in the Chapter 4 before being processed and analyzed by nano-LC-MS/MS.

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Samenvatting

Matrixmetalloproteïnases (MMP's) zijn bij een groot aantal pathologische processen betrokken die gerelateerd zijn aan een verstoring van hun proteolytische activiteit. Daarom is de meting van actieve MMP's nuttiger voor de karakterisering van biologische of pathologische voorvallen, dan de bepaling van het totale overschot.

Het doel van dit PhD-project was de ontwikkeling van analytische technieken ter controle van MMP-activiteit. Daarvoor is onderzoek gedaan naar fotogevoelige labeling , absolute kwantificering met massaspectrometrie in de Selected Reaction Monitoring (SRM) modus, en naar op activiteit gerichte verrijkingstechnieken.

Het labelen met een S1' fotogevoelige, op activiteit gerichte probe en het katalytisch domein van recombinant MMP-12, resulteerde in een verhouding van 1:1 (**hoofdstuk 2**). Daarnaast kon het gelabelde eiwit geconcentreerd worden met monomere avidine deeltjes, gebaseerd op de biotine keten die deel uitmaakt van de probe. De karakterisering van de aminozuur(en) die vastgehouden worden door covalente binding(en) met de kunstmatige probes wordt nog onderzocht.

Door de toepassing van LC-MS/MS (SRM modus) is er een doelgerichte analyse van MMP-9 in bronchoalveolaire lavage vloeistof (BALF) ontwikkeld (**hoofdstuk 3**) . Deze SRM analyse maakt de absolute kwantificering van MMP-9 naar het lage ng / mL niveau mogelijk. De ontwikkelde analyse werd aangepast voor MMP-12 door het meten van een andere set van signatuurpeptiden.

Met behulp van deze bepaling wordt de activiteit van MMP-9 nog nietgeanalyseerd, Daarom werd de op activiteit gerichte opzuiverbenadering gecombineerd met de MMP-9 en MMP-12 SRM analyse (**hoofdstuk 4**). We hebben deze methodologie verder uitgebreid door de peptiden die behoren tot het pro-domein van MMP-9 alsmede die van de belangrijkste endogene remmer van MMP-9, TIMP-1 te meten.

Tenslotte worden de voor- en na-delen van de ontwikkelde technieken en hun potentieel voor automatisering besproken (**hoofdstuk 5**).

List of publications

Geurink, P. P, Klein, T, Prely, L, Paal, K, Leeuwenburgh, M. A., van der Marel, G. A., Kauffman, H. F., Overkleeft, H. S., Bischoff, R., Design of Peptide Hydroxamate-Based Photoreactive Activity-Based Probes of Zinc-Dependent Metalloproteases. European Journal of Organic Chemistry 2010, 11: 2100-2112.

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